The Influence of Male Diet on Life History Traits of Female Mosquitoes

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Abstract

In the field, mosquitoes characteristically feed on sugars soon after emergence and intermittently during their adult lives. Sugar meals are commonly derived from plant nectar and homopteran honeydew, and without them, adults can only survive for a few days on larval reserves. In addition to sugar, females of most species rely on blood for the initiation and maintenance of egg development; thus their reproductive success depends to some extent on the availability of blood hosts. Males, on the other hand, feed exclusively on sugars. Consequently, their sexual maturation and reproductive success is largely dependent upon access to sugar sources. Plant nectar and homopteran honeydew are the two main sugar sources utilized by mosquitoes in the wild. Previous laboratory studies had shown that differences between nectar sources can affect the survivorship and biting frequency of disease vectoring mosquitoes. However, little is known on how sugar composition influence the reproductive processes in male mosquitoes. Male mosquitoes transfer accessory gland proteins and other hormones to their mates along with sperm during mating. In the female, these seminal fluid constituents exert their influence on reproductive genes that control ovulation and vitellogenesis. The present study tests the hypothesis that the mates of males consuming different sugar meals will exhibit varying levels of induction of vitellogenin (a gene which regulates the expression of egg yolk precursor proteins). Real-time quantitative RT-PCR was used to investigate how each sugar meal indirectly influences vitellogenin mRNA abundance in female *Anopheles stephensi* following mating. Results indicate that mates of nectar-fed males exhibit 2-fold greater change in vitellogenin expression than the mates of honeydew-fed males. However, this response did not occur in non-blood fed controls. These findings suggest that the stimulatory effect of mating on vitellogenesis in blood meal-reliant (i.e. anautogenous) mosquitoes may only be synergistic in nature.

The present study also sought to compare the potential fitness costs of mating incurred by females that do not necessarily require a blood meal to initiate a reproductive cycle (i.e., exhibit autogeny). Females of the facultatively autogenous mosquito, *Culex molestus* were allowed to mate with males sustained on either nectar or honedyew. Mean lifetime fecundity and survivorship of females under the two different mating regimes were then recorded. Additionally, one-dimensional gel electrophoresis was used to verify the transfer of male accessory gland proteins to the sperm storage organs of females during mating.While there was no significant difference in survival between the test treatments, the mates of nectar-fed males produced 11% more eggs on average than mates of honeydew-fed males. However, additional data are needed to justify the extrapolation of these findings to natural settings. These findings prompt further investigation as the differences caused by diet variation in males may be reflected across other life history traits such as mating frequency and insemination capacity.

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20E.....	**20**-Hydroxy**e**cdysone
AG……	**A**ccessory **G**land
AGP.....	**A**ccessory **G**land **P**rotein
AN.....	**A**rtificial **N**ectar
AH.....	**A**rtificial **H**oneydew
AsVg1.....	***A**nopheles **s**tephensi **V**itello**g**enin **1***
AsRPS6.....	***A**nopheles **s**tephensi **R**ibosomal **P**rotein **S6***
BF.....	**B**lood **F**ed
JH.....	**J**uvenile **H**ormone III
NBF.....	**N**on **B**lood **F**ed
NER.....	**N**ormalized **E**xpression **R**atio
Vg.....	**V**itello**g**enin
WNV.....	**W**est **N**ile **V**irus
YPP.....	**Y**olk **P**rotein **P**recursor

CHAPTER 1

Literature Review

Males and their life history traits are frequently overlooked in studies regarding the reproductive ecology of disease-vectoring Diptera. This is mainly because it is the females that facilitate the spread of pathogens. In recent years however, the search for innovative population control strategies has led to increased interest in the reproductive ecology of males. These approaches have been sought to improve our understanding of the underestimated role of males in population growth and may potentially be exploited for control purposes. The literature reviewed in this chapter will highlight different aspects of the life history of the mosquito but will focus primarily on the interplay between sugar feeding and recently published works on male and female reproductive ecology.

1. Sugar feeding behaviour

1.1.1 Incidence of sugar feeding

Reports of mosquito sugar feeding in the field are based either on behavioural observations or on the analysis of midgut contents. Behavioural studies that take into account the relative availability of all possible sugar hosts are still prone to misinterpretation. In the field it is difficult to distinguish sugar feeding behaviour from other behaviours that do not result in ingestion of sugar, such as resting and probing. Additionally, this technique is confounded by the fact that sugar feeding occurs rapidly and its frequency tends to vary depending on the time of day and the proximity of a given sugar source. Sugar feeding from nearly inconspicuous sources like honeydew is also difficult to ascertain using this technique (Mahmood and Reisen, 1982). Unlike direct observations, gut content analyses are not easily misinterpreted.

Gut sugars are commonly detected using the cold-anthrone test (van Handel, 1972) and various forms of chromatography (Heimpel and Jervis, 2004). Anthrone tests allow researchers to distinguish between fructose and other sugars. Since fructose is absent in the hemolymph it serves as a marker for ingested sugar in whole body preparations (van Handel, 1984). One limitation of anthrone tests is that they cannot be used to determine the origin of different sugar meals. In the wild, mosquitoes may rely on plant nectar, honeydew, plant exudates and fruit juices so these tests are of little use in studies assaying the range of sugar sources utilized by mosquitoes. For increased sensitivity, researchers rely on chromatographic techniques (e.g. gas chromatography, thin layer chromatography and high-performance liquid chromatography) which can not only be used to detect digested and undigested sugar meals but also varying levels of sugars distinctive to a given sugar source (Burkett *et al.* 1998, Clements, 1999).

Some species also utilize healthy plant tissue to acquire sugars and other nutrients from the phloem sap (Schlein and Müller, 1995). Evidence for this type of behaviour comes from the detection of stained cellulose (indigestible marker) in the midgut of wild caught mosquitoes (Schlein and Müller, 1995). Analysis of chloroplast DNA found in the midguts of wild-caught mosquitoes has even been used to deduce plant-host preference (Junnila *et al.*, 2010).

One drawback common to all types of gut content analyses is that most sugars have short half-lives following ingestion. In one study, only 214 out of 403 wild-caught mosquitoes tested positive for the presence of sugar (Russell and Hunter, 2002). Among those that tested positive for sugar only ~8% tested positive for honeydew-specific sugars. Additionally, sugar positivity rates are subject to intra-specific variation based on the age and sex of the individual mosquito. Consumption and digestion rates will also vary depending on maturation level, extent of mating and flight activities, ambient temperature, abundance of the food source etc. As a result many individuals will test negative despite having recently sugar fed. The results of

those that test positive therefore, do not necessarily reflect the sugar feeding behaviour of the majority of the population.

1.1.2 Timing and frequency of sugar feeding

The timing and frequency of sugar feeding depends on two factors: (1) sex of the individual and (2) reproductive strategy of the species. Males of all species will take sugar meals throughout their adult life. A field study by Yuval *et al.* (1994) demonstrated that male *Anopheles freeborni* sugar fed after swarming activity (*discussed later*) ceased and before males entered resting sites. By comparing the amount of fructose detected in resting males caught in the morning and those caught in the afternoon they concluded that sugar feeding takes place in the night after swarms disperse. Yuval *et al.* (1994) also demonstrated the frequency of sugar feeding in the population by examining the levels of nectar sugars of individual males sampled before and after a 20 minute swarming bout. The absence of nectar in males collected after swarming indicated that they do not leave the swarm to sugar feed and subsequently resume swarming.

Females of anautogenous species (i.e., those that require a blood meal to produce eggs) tend to take sugar meals after they have begun digesting a blood meal, while they are gravid or between oviposition and the subsequent blood meal (Gary and Foster, 2006). Müller *et al.* (2010) conducted a field study of *An. gambiae* and demonstrated that the times of sugar seeking and blood seeking, though to some extent overlapping occurred in distinctly different times in the diel cycle. They found that attraction of females to flowers peaked in the early evening and early morning, whereas attraction to human odour occurred mainly around midnight.

As mentioned earlier, most of these estimates are based on the detection of sugar meals present at varying stages of digestion in sampled individuals. But because they vary between different species and are influenced by temperature fluctuations, host preference and

abundance, it is difficult to establish general trends in sugar feeding activity. Based on the literature reviewed, males in the field during warm weather may take sugar approximately every 1-2 days. Host-specificity in females seems to dictate their dependence on sugar for survival and reproduction. Females of anthropophilic species (i.e., show preference for human blood) may take just one sugar meal every 6-9 days (Harrington *et al.*, 2001, Kaufmann and Briegel, 2004, Fernandes and Briegel, 2005). While females of zoophilic species (i.e., show preference for animal blood) will often contain undigested sugar and may sugar feed as often as once every 3-4 days. Without sugar, they can die rapidly, despite frequent access to animal blood (Nayar and Sauerman, 1971, Fernandes and Briegel, 2005).

1.1.3 Composition of different sugar meals

Mosquitoes obtain sugar from a variety of sources. The most commonly utilized sources are floral and extrafloral nectaries and honeydew (Magnarelli, 1977; Mogi and Miyagi, 1989; Foster, 1995; Gary and Foster, 2004). Occasionally mosquitoes will consume sugar from damaged or decaying fruit and seeping phloem sap from plant wounds (Foster, 1995).

Nectars are floral or extrafloral secretions derived from phloem sap. They are primarily solutions of the disaccharide sucrose and its hydrolysis products fructose and glucose (Kevan and Baker, 1983; Baker and Baker, 1983; Wäckers, 2001). Several glycosidases have been reported in mosquito saliva (Marinotti *et al.* 1990) and the midgut (Souza-Neto *et al.* 2007) that cleave sucrose into its constituents. The nectar secretions of certain plants may also contain relatively lesser concentrations of sugars such as the monosaccharides galactose, mannose, rhamnose, the disaccharides maltose, melibiose, and traces of the trisaccharide raffinose (Wäckers, 2001). In addition to sugars, traces of amino acids, and organic and inorganic micronutrients (e.g. antioxidants, alkaloids, vitamins and minerals) may also be present (Baker and Baker, 1983). Although trace amino acids alone do not stimulate or support egg

development they have been shown to increase the longevity of female *Culex quinquefasciatus* mosquitoes (Vrzal *et al.* 2010). Given that sugars are not volatile (Foster, 1995), these accessory constituents presumably serve as insect attractants resulting from the pollination syndrome (Jhumur *et al.* 2008).

Honeydew is a semi-liquid excretion derived from phloem-feeding members of the order Homoptera (Wäckers, 2001; Hendrix *et. al*., 1992). Among the members of this order are aphids (Aphididae) and coccids (Kermidae) (reviewed in Rabb, 1984). The alimentary tract in these insects has a modification known as a filter chamber that concentrates nutrients in the midgut and small intestine. This allows excess water (containing some sugar and waste materials) to bypass the midgut and small intestine, and be exuded from the rectum as honeydew which collects on the insects' resting sites (e.g. top surface of leaves) (Rabb, 1984; Chapman, 1998).

The fact that sucrose is virtually the only sugar present in the phloem sap of most plants suggests that the Homopteran produces the various oligomers in honeydew by isomerization and polymerization of sucrose (Ziegler, 1975; Hendrix *et al.*, 1992). These conversions are carried out fairly efficiently since sucrose constitutes only about 25% of the sugar in honeydew (as cited in Hendrix *et al.*, 1992). Glucose, fructose, galactose, trehalose, maltose, raffinose, and erlose constitute, in varying proportions, the remaining 75 % of the sugar in honeydew. In addition to these sugars some homopterans secretions contain the honeydew-specific sugars, melezitose (a trisaccharide) and stachyose (a tetrasaccharide) (Wäckers, 2001). Hendrix *et al.* (1992) reported that the sugar composition of excreted honeydew will vary depending on the homopteran species and not just the species of plants upon which the homopterans utilize. For example, honeydew from the cotton aphid (*Aphis gossypii*), reared on cotton was found to contain 40% melezitose (i.e., 40% of total sugar), while that secreted by cotton-reared whiteflies (*Trialeurodes vaporariorum*) was found to contain 20% turanose (a disaccharide)

and only minute amounts of melezitose (Hendrix *et al.*, 1992). In addition to carbohydrates, a host of other organic substances including amino acids, organic acids, and phytohormones may also be present in homopteran honeydew (Auclair, 1963).

1.1.4 Diet preference

It is common practice to provision mosquito colonies with sugar solutions including sucrose or glucose in the place of natural sugars to allow mosquitoes to thrive in insectaries (Gillett *et al.* 1962; Grimstad and Defoliar, 1974; Benedict 2007). In the field, mosquitoes are faced with a wide range of sugar meal options. There have only been a few comprehensive reports on sugar source preference by males in nature. Abdel-Malek and Baldwin (1961) found that *Aedes aegypti* and three indigenous Canadian mosquitoes (*Ae. punctor*, *Ae. diantaeus* and *Ae. implacibilis*) fed on only 3 of 24 native plant species offered to them. A similar field study revealed that *An. sergentii* males exhibited plant-host preference. Males of this species used only 3 out of 40 plant species for sugar meals (Abdel-Malek, 1964). Dual choice olfactory bioassays have been used to show that this preference is most likely driven by odour cues (Lefèvre *et al.*, 2009; Gouagna *et al.*, 2010).

Interestingly, males and females of some species exhibit varying degrees of responsiveness to plant odours. Müller *et al.* (2011), found that female *Ae. albopictus* exhibited greater attraction to certain flowering ornamental plants (e.g. deciduous shrubs) than to flowering wild plants (e.g. perennial shrubs), seed pods, fruits, and even aphid honeydews. On the other hand, males found wild flowers to be most attractive. Neither sex exhibited an attraction to foliage soiled with honeydew excretion of three different aphid species, though they would readily imbibe honeydew when encountered (Müller *et al.*, 2011). Similar observations were reported in *Cx. pipiens* (Schlein and Müller, 2008), as branches of bait plants with and without honeydew did not differ in the number of mosquitoes attracted. This suggests

that honeydew itself is not an attractive sugar meal and that honeydew feeding is likely an opportunistic behaviour observed when mosquitoes land on soiled vegetation (Foster, 1995). One variable that was not considered in the previous studies (Müller *et al.*, 2011; Schlein and Müller, 2008) is the age of the honeydew. In nature, honeydew contaminated with microorganisms would produce fermentation products that could enhance the odour of otherwise odourless excretions. The attraction to fermentation products is reasonable to expect since mosquitoes given equal opportunity are more attracted to decomposing fruits than fresh undamaged fruits of the same plant species (Müller *et al.*, 2011). In addition to olfactory stimuli, diet preference in mosquitoes is likely driven by gustatory cues, as sugars themselves are not volatile. In adults these cues are detected by taste receptors located on their tarsi and mouthparts (reviewed in Ignell *et al.*, 2010). Floral fragrances, in particular contain a host of compounds including phenols, aldehydes, fatty acid derivatives and benzenoids which may attract mosquitoes (Foster, 1995; Qui and van Loon, 2010).

In a dual choice assay, both sexes of *Ae. aegypti* preferred sucrose over turanose, fructose and glucose (Ignell *et al.*, 2010). When sucrose utilization was compared with an equimolar blend of glucose and fructose, neither sex was able to differentiate between the disaccharide and its monosaccharide constituents. In contrast, their behavioural response to individual amino acids indicated that both sexes are able to differentiate among the various amino acids, but only when the amino acids were served in a solution containing sucrose (Ignell *et al.*, 2010). Male and female *Ae. aegypti* were differentially responsive to certain amino acids: females exhibited preference for sucrose solution containing leucine, threonine and phenylalanine, whereas males preferred sucrose containing glutamate. This variation in amino acid preference may be the result of varying reproductive investments of each sex (Ignell *et al.*, 2010). Male mosquitoes rely entirely on larval energy reserves and sugar-rich diets to

produce gametes, while female *Ae. aegypti* must feed on blood to initiate and complete a reproductive cycle (Foster, 1995; Ignell *et al.,* 2010).

It is important to note that the diet presentation in laboratory based studies like those in Ignell *et al.*, (2010) do not reflect the natural environment of the mosquito because the potential natural sugar sources are not always found in close proximity. Additionally, foraging behaviour of wild mosquitoes is not restricted by limited enclosure space. Nevertheless, single- and dual-choice assays do provide relevant insight regarding the acceptance (or rejection) by mosquitoes of different compounds found in natural sugar sources (Foster, 1995; Ignell *et al.*, 2010).

1.2 Sugar utilization

The caloric value of larval-acquired energy reserves (mainly in the form of glycogen and lipids) that is available to adults at emergence ranges between 0.1 and 2.9 cal (at least in some male and anautogenous female mosquitoes, Magnarelli, 1983; Magnarelli, 1986; Magnarelli and Andreadis, 1984; Magnarelli and Andreadis, 1987). Once these energy reserves have been depleted, adults must turn to sugar and/or blood meals to provide the energy required for survival, host seeking, mating, egg development, oviposition etc. Among the most extensively studied of these are longevity, flight, and reproduction.

1.2.1 Longevity

There is a trade-off between somatic maintenance and reproductive investment, thus the effect of sugar meals on longevity is estimated best in laboratory settings where individuals can be isolated immediately following emergence to prevent mating. Newly emerged adults provisioned with water alone in the laboratory can survive for only a few days on their energy reserves (in *An. stephensi*, Abraham, personal observation). This coincides with a number of reports among several other genera (Nayar and Sauerman, 1975; Nayar and Pierce, 1977;

Nayar and Pierce, 1980; Andersson 1992). Sugar deprived males typically die within a few days. This is even true of females with frequent access to blood, presumably, because larval reserves are insufficient for blood protein metabolism (Foster, 1995).

The nutritional value of sugar meals with respect to their effect on mosquito survival varies among different nectar and honeydew sources. Gary and Foster (2004) reported that female *An. gambiae* survive longer on the extra-floral nectar of the extensively cultivated, cassava plant than those sustained on shrubs (e.g. extra-floral nectar of castorbean and lantana plants), as well as mealybug honeydew. This was observed even though the amount of sugar obtained from each source was similar. That their mean survival times differed significantly, suggests that other components or the sugar composition of cassava nectar positively affected mosquitoes. Cassava is an important crop in tropical and subtropical regions of Africa, Asia, and South America, and is incidently found near human dwellings where *An. gambiae* breed and seek blood meals. Similar differences were also found in a study in Kenya, where eight local plant species were tested for their ability to enhance *An. gambiae* survival (Impoinvil *et al.,* 2004). Of the different plants studied, only castor bean increased survival to an extent comparable to a 6% sucrose solution. On the remaining plant species (e.g. lantana and sweet potato) mosquito survival times either did not differ from, or were slightly worse than observed in water fed controls (Impoinvil *et al.*, 2004). Yet another study found that female *Cx. quinquefasciatus* provided nectar mimics containing mixtures of sugar and amino acids, survived 5% longer than those sustained on nectar mimics alone (Vrzal *et al.*, 2010). Interestingly, this benefit to survival was not observed in males when fed a similar diet. These studies demonstrate that there can be considerable variation in the nutritive quality of different sugar sources; even from within the same plant community (Impoinvil *et al.*, 2004).

The nutritive value of a sugar meal also depends on its concentration. Sugars that are dry must first be solubilized with saliva before they can be ingested (Nayar and Pierce, 1980).

Female *Ae.aegypti* exhibit extended lifespan with access to dilute sugar sources (<0.5-2%), while highly concentrated sources can not be utilitzed as efficiently despite their caloric content (e.g. dry solid sugar) (Nayar and Pierce, 1980; Briegel *et al.*, 2001). However, dilute sugar meals induce a caloric deficiency that causes glycogen and lipid reserves to become progressively lower as the females age (Briegel *et al*., 2001). Andersson (1992) compared survivorship of female *Ae. communis* of different sizes and with access to different concentrations of sucrose or fructose. While no difference in survivorship was found between sucrose and fructose diets, the mean survival time was shorter on 10% solutions than on the higher concentrations. These observations raise an interesting question. Can mosquitoes with unrestricted access to dilute or highly concentrated sugars compensate for the quality of their meals by adjusting their feeding frequency? Most laboratory-based studies make the assumption that mosquitoes will increase their feeding frequency when granted *ad libitum* access to dilute sugar meals.

1.2.2 Flight

For both sexes ingested sugars are the primary substrate for flight. There is a direct correlation between ingested sugars and flight range. This has been observed across a number of genera and is based on experiments where individual adults are tethered to flight mills (reviewed in Foster, 1995). Direct comparisons of the consequences of nectar and honeydew feeding by mosquitoes on flight activity have not been investigated. However, honeydew feeding has been shown to increase flight distance and duration in female black flies in a dose-dependent manner (Stanfield and Hunter, 2009). This review will focus mainly on the sex-specific types of flight activity.

Typical anopheline males may mate 0-3 times in their lifetime, at least in monandrous species with a 1:1 sex ratio (Howell and Knols, 2009). In order to achieve mating success,

males must first survive to sexual maturity. Males typically reach sexual maturity between 4-5 days post emergence (Mahmood and Reisen, 1982; Huho *et al.*, 2006; Pondeville *et al.*, 2008) and their outward appearance is characterized by the presence of rotated terminalia and erect antennal fibrillae (Howell and Knols, 2009). Following emergence males engage in the energetically costly behaviour of swarming.

In most species swarming occurs at dusk or both dusk and dawn (Howell and Knols, 2009). Some *Aedes spp.* swarm during the day, with swarming bouts lasting anywhere from 10 minutes to over an hour (Nielsen and Nielsen, 1958; Yuval *et al.*, 1994). The swarm itself is stationary (normally over a body of water), but participating males are in constant movement (Nielsen and Nielsen, 1958), until a female is encountered and mating ensues. The ability to mate improves in males during its first week of adult life (Verhoek and Takken 1994) presumably due to the recruitment of adequate nutrient reserves from sugar meals. It follows then, that the ability of male mosquitoes to locate sugar meals throughout their life contributes to their reproductive success.

Yuval *et al.* (1994) found that *An. freeborni* feed on nectar only after swarming in the evening, as only resting males collected in the morning tested positive for fructose, whereas males collected in the late afternoon or during swarming did not. The amount of sugars and glycogen found in males that had recently engaged in swarming was significantly lower than in their resting counterparts. The energetic cost of swarming was calculated to be 0.39-0.51 calories per hour and males engaged in a ~40 minute swarming bout consumed over 50% of their available reserves (Yuval *et al.* 1994). This finding coincides with an earlier work with *Cx. tarsalis* Coquillett (Reisen *et al.* 1986). Interestingly, lipids derived from sugar meals were not used in flight, as swarming and resting males possessed similar lipid contents (Yuval *et al.* 1994). It is likely that lipids may be used during less energetically-taxing activities like resting.

These data suggest that natural selection should always favour males that take sugar meals and thereby greatly increase their mating potential and competitive abilities.

Unlike males, females need sugar and to some extent lipids to fuel a wider variety of goal-oriented flights, due to their relatively greater reproductive investment (e.g. seeking hosts and oviposition sites). A recent laboratory study by Kaufmann and Briegel (2004) demonstrated that both sugars and lipids can be utilized by female *An. gambiae* Giles during long flights. Lipids comprised about half of the flight substrate utilized by these females. Whether it's the duration or strenuousness of a given behaviour that induces the switch to lipid metabolism is not known. However, this ability to mobilize lipid reserves appeared to be subject to interspecific variation as females of *An. atroparvus* utilized significantly less lipids (i.e., only a third) for flight. Sugar meals can also determine the duration of host seeking behaviours in some anautogenous species. Nasci (1991) reported that sugar fed *Ae.aeygpti* females were more persistent at obtaining a blood meal from an irritable host (judged by total number of landing attempts) than sugar deprived ones. These reports show how sugar meals could have huge implications for reproductive success indirectly through their influence on flight activity alone. The direct effects of sugar feeding on reproduction are even more profound.

1.2.3 Reproduction

Although there is a large body of evidence demonstrating the direct effects of sugar feeding on females' reproductive physiology and behaviour, interest in male reproductive processes is lacking. For females the reproductive strategy of the species dictates the extent of their reliance on sugar.

In the case of females of autogenous species (i.e., those not requiring a blood meal for initial egg development), a sugar meal often is necessary for the development of the first batch

of eggs (O‘Meara 1985, 1987). A few species are exclusively autogenous and feed only on sugar. In contrast, females of obligate anautogenous species rarely contain undigested sugar (Edman *et al.*, 1992; Costero *et al.*, 1998; Beier, 1996; Spencer *et al.*, 2005) relying entirely on blood meals to complete oogenesis. However, even among anautogenous mosquitoes there are species that rarely or never seek blood until they take at least one sugar meal (Renshaw *et al.*, 1995; Briegel *et al.* 2001). This initial sugar meal replenishes the females’ energy reserves and is necessary for the initiation of ovarian follicle development. Once initiated the acquisition of a blood meal induces the accumulation of yolk into the maturing eggs (discussed below) (Attardo *et al.*, 2005). Sugar meals taken before the initiation of an ovarian cycle can increase the number of eggs in a clutch by enhancing energy reserves. This effect has been observed in both anautogenous and autogenous species (O'Meara, 1985; Nayar and Sauerman, 1971, 1975). In the *Ae.communis* sugar feeding during an ovarian cycle has even been shown to promote the rate of egg development (Andersson, 1992).

In the laboratory, with unrestricted access to sugar, females of some species exhibit decreased blood feeding frequency and delayed oviposition (Hudson, 1970; Foster and Eischen, 1987). The former could be due to limited space in the crop or repleted energy reserves. In female *An. stephensi* 24 hour sugar deprivation is known to increase the likelihood of blood feeding (Abraham, unpublished observation). Why sugar induces delayed oviposition is not known but the duration of these delays appears to be directly proportional to concentration of sugar provided (Foster and Eischen, 1987).

Sugar feeding may also influence offspring quality through a maternal effect. Fernandes and Briegel (2005) found that sugar fed *An. gambiae* females invested slightly more calories in different protein-to-lipid proportions into their eggs, than their blood fed counterparts. Although they did not compare the development of the larva, it is possible that under some selective pressure in the field these differences could translate into fitness benefits for sugar fed

individuals. Interestingly, in certain anautogenous female species, sugar deprivation after the completion of egg development results in eggs with depleted glycogen deposits (van Handel, 1992). This is likely part of a stress coping mechanism which reduces reproductive investment to improve somatic maintenance.

Along with the status of energy reserves, the availability of sugar appears to play a role in the expression of autogeny in some species. In autogenous species the accumulation of nutrients in the larval stage is regulated by environmental and genetic factors. In adults, energy from these reserves and/or sugar meals is used for the activation of egg development. On the other hand, anautogenous mosquitoes appear to maintain relatively low levels of stored nutrients (Attrado *et al.*, 2005), which most likely prevents egg development. This suggests that nutrition plays a huge role in the regulation of reproduction in mosquitoes. The nutritional deprivation that occurs in anautogenous mosquitoes is lifted when these mosquitoes blood feed, thereby inducing egg development. The fact that many species have both autogenous and anautogenous strains and that autogenous mosquitoes can be generated from obligatorily anautogenous mosquitoes (Lea, 1964; Trpis, 1977), suggests that the mechanism for regulation of egg development is considerably plastic in autogenous and anautogenous mosquitoes. In order to understand the role of nutrition in the regulation of egg development a deeper look at the process of vitellogenesis is necessary.

1.3 Vitellogenesis

Mosquitoes, like all oviparous insects, provision their eggs with vitellin -- the major yolk protein in eggs. Vitellogenesis is the large-scale synthesis and secretion of yolk protein precursors (YPPs) (Attrado *et al.*, 2005; Tufail and Takeda, 2008). The main YPP genes activated during vitellogenesis are vitellogenin (*Vg*), vitellogenic carboxypeptidase, vitellogenic cathepsin B and lipophorin (Attrado *et al.*, 2005). The most highly expressed of

these genes is *Vg*, the precursor of vitellin (Raikhel *et al.*, 2002; Attrado *et al.*, 2005). Before YPPs can be synthesized, the abdominal fat boy must first become competent to respond to signals that initiate vitellogensis (Raikhel *et al.*, 2002, Attrado *et al.*, 2005). Fat body cells have a wide range of functions including energy storage and metabolism, protein synthesis and a number of endocrine functions (Attrado *et al.*, 2005). In females of anautogenous species, the fat body begins to undergo changes that make it responsive to signals that induce vitellogenesis upon emergence from the pupae stage (Redfern, 1982; Attrado *et al.*, 2005). These changes include a peak in juvenile hormone III (JH), (Flanagan and Hagedorn, 1977; Raikhel and Lea, 1990; Hagedorn, 1994) which makes the fat body competent to respond to the steroid hormone 20-hydroxyecdysone (20E) and to synthesize the massive amounts of yolk protein required for egg maturation (Raikhel and Lea, 1983). Once the fat boy has been primed for 20E, it enters a reproductive state of arrest characterized by the repression of the YPP genes. This state of arrest is alleviated upon blood feeding which marks the beginning of vitellogensis.

The accumulation of the *Vg* mRNA in the fat body of blood fed females varies in a species-specific manner. In females of *Ae aegypti, Vg* mRNA appears 1 hour following a blood meal. Afterward titres increase steadily, reach a peak between 24 and 36 hours, and then decline to basal levels by 48 hours post blood meal (Racioppi *et al.*, 1986). Racioppi *et al.* (1986) noted that the pattern of *Vg* mRNA accumulation after a blood meal was closely paralleled by the pattern of YPP synthesis, as well as, free 20E titres in whole body preparations. This study which was based on a hybridization assay coincides with the immunohistochemical data of Raikhel and Lea (1983) who worked with another strain of *Ae. aegypti*. Using a radiolabelling-based approach, Redfern (1982) followed the synthesis of 3 vitellogenic proteins in recently blood fed *An. stephensi* and noted that while the accumulation of the 3 yolk proteins was undetectable at 4 and 8 hours post-blood feeding, it changed drastically around 12 hours post blood feeding. Unfortunately, the qualitative nature of

Redfern's (1982) study prevents one from making interspecies comparisons regarding the pattern of vitellogenin expression between *An. stephensi* and *Ae. aegypti*. Blood feeding also stimulates an increase in JH esterase activity and inhibition of JH production, causing a rapid decrease in the basal hemolymph JH titer (Attardo *et al.*, 2005).

The activation cascade for vitellogensis starts with the blood meal induced secretion of the peptide hormone, ovarian ecdysiotropic hormone (OEH) from the medial neurosecretory cells in the brain (Lea, 1967; Attardo *et al*., 2005). This hormone in turn stimulates production of the steroid hormone ecdysone in the ovaries, which travels to the fat body and is hydroxylated to 20E. 20E is the major stimulus that up-regulates YPP gene expression in mosquitoes (Hagedorn, 1985; Attrado *et al.*, 2005). After activation by 20E, YPP genes are transcribed and translated specifically in the fat body. The YPP are then processed and secreted from the fat body into the hemolymph, from where they are taken up by the developing oocytes via receptor-mediated endocytosis. After incorporation into oocytes these yolk proteins are stored in crystalline form, providing nourishment for the future embryo. According to Hagedorn (1985), 20E titers typically increase and peak at 24 hours post blood meal. At around 36 hours post blood meal, the fat body reverts back to a nutrient storage and metabolism function until the next vitellogenic cycle is initiated. The cessation of YPP synthesis in the fat body marks the last stage of vitellogenesis (Raikhel *et al.*, 2002). To date, blood feeding is the only known stimulus to activate vitellogenesis in anautogenous mosquitoes. However, in recent years there has been growing interest in certain components of male-derived seminal fluid that have been implicated in triggering responses in several processes related to female fertility.

1.4 Exogenous modulators of female reproduction

Male-derived seminal fluids are a mixture of proteins and other molecules (Avila *et al*., 2011). Proteins in the seminal fluid are mainly the products of male secretory tissues called accessory glands (AGs, Figure 1.5). These accessory gland proteins (AGPs) are transferred to females along with sperm during mating (Wolfner, 2009). They are major effectors of a wide range of female post-mating responses, including changing female likelihood of remating, increasing ovulation and egg-laying rates, changing female flight and feeding behavior, inducing antimicrobial activities, and modulating sperm storage parameters (Wolfner, 2009; Avila *et al*., 2011). The absence of AGPs from the ejaculate adversely affects the fitness of both sexes (Avila *et al*., 2011). AGPs identified to date represent numerous protein classes, including proteases/protease inhibitors, lectins, prohormones, peptides, and protective proteins such as antioxidants (Avila *et al*., 2011). These protein classes are most extensively studied in *Drosophila* but their presence has been reported in the ejaculate of many organisms, ranging from arthropods to mammals (reviewed in Poiani, 2006; Wolfner, 2009). While nonprotein molecules are also present in seminal fluid (e.g., steroid hormones in mosquitoes: Pondeville, 2008), research on the effects of seminal fluid receipt has focused largely on the action of AGPs.

Modulation of female post-mating gene expression has been examined in *D. melanogaster* and, to a lesser extent, in *An. gambiae*. AGP function has generally been elucidated by: (1) the injection of a purified AGPs or protein fractions into virgin females, (2) biochemical analysis, (3) the removal of putative AGPs by RNAi or mutation in *Drosophila*, and (4) ectopic expression of AGPs in virgin *Drosophila* females (Wolfner, 2009, Avila *et al*., 2011). Direct methods have also identified the roles of specific AGPs in processes such as gene regulation and physiological processes such as sperm storage (Rogers *et al*., 2008).

In *An. gambiae* females, RNA transcript abundance for 141 genes experience changes at 2, 6, and 24 h post-mating (Rogers *et al*., 2008). The number of genes with changes in expression levels two-fold or greater increased with time since mating. Changes in transcript abundance of many of these genes (which were predicted proteolysis regulators) persisted for at least 4 days post-mating (Rogers *et al*., 2008). Interestingly, in the spermatheca, a predicted vitellogenic gene was also highly upregulated following mating (Rogers *et al*., 2008).

Preface

The primary objective of the following experiments is to compare nutritional value of nectar and honeydew with regard to male mating success. Given that there are several life history traits that reflect their mating success, a preference for a given sugar source may potentially manifest directly as the males reach sexual maturity (e.g. higher insemination rates, increased sperm production and accessory gland secretions) or indirectly, in their mates (e.g. increased egg production and reduced mate receptivity).

Chapter 2 investigates the effect of male nutritional history on a key stage in mosquito egg production called vitellogenesis. It is the large-scale synthesis and secretion of yolk protein precursors in the fat body. Different species have developed variations of the requirement of blood for the initiation of vitellogenesis. Mosquitoes capable of undergoing the first gonotrophic cycle without a blood meal are termed autogenous, while those that do not are considered anautogenous. In this experiment, the expression of the *vitellogenin, Vg*, in recently-mated females is compared (using a real-time RT-PCR assay) when their mates have been provisioned either nectar or honeydew. *Anopheles stephensi*, a malaria vector in many parts of Asia, was used as a model in this experiment because of its medical importance and the availability of its sequence data.

There are certain species which remain medically relevant despite having adopted a reproductive strategy that is not limited by the need to blood feed. One example is the West Nile Virus (WNV) vector, *Culex molestus.* In such species, females can develop their first batch of eggs autogenously, but then require blood for subsequent egg batches. Because the mosquito life cycle can essentially be complete in the absence of blood hosts, blood is less of a limiting resource in these models. Thus, other modulators of egg development can effectively be examined in the absence of nutritional stimuli.

In addition to a blood stimulus, the mating status of the female has been shown to have an effect on their ability to develop eggs. In *Aedes taeniorhynchus*, autogenous egg maturation does not begin until the female has mated (O'Meara and Evans, 1976). It has since been demonstrated that substances from the male accessory gland delivered with the sperm are responsible for this stimulus. Thus, in Chapter 3 autogenous egg production of female *Cx. molestus* is compared following a single mating to males with varying nutritional histories. The accessory gland protein profiles of males under each diet regime (i.e., nectar or honeydew) were also compared by 1D gel electrophoresis.

Access to food resources is vital for a female's survival and reproductive success. In environments where resources (e.g. blood or sugar hosts) are limited, trade-offs between fitness-related traits (e.g. somatic maintenance and reproductive investment) arise. Considering the divergent interests of males and females in reproduction, mating may potentially shift this trade-off in favor of increased reproductive investment, especially if environmental resources are plentiful. Consequently, females may suffer deleterious effects, such as reduced survivorship, as a result of mating. To test this hypothesis, the survivorship of females under varying mating regimes was also compared in the *Cx. molestus* model.

CHAPTER 2

Influence of male nutritional history on vitellogenin expression in the anautogenous mosquito, *Anopheles stephensi* (Diptera:Culicidae)

2.1 Introduction

Anautogeny is a successful reproductive strategy utilized by many mosquito species and other disease-transmitting arthropod vectors. Malaria infects between 300–500 million people and kills roughly one million people (mostly young children) worldwide each year (CDC, 2003). Developing an understanding of the mechanisms underlying anautogeny in mosquitoes is very important because this reproductive strategy is the driving force behind the transmission of disease to millions of people. Among the most extensively studied regulators of vitellogensis are the hormonal and nutritional control mechanisms.

2.1.1 Hormonal regulation of vitellogensis

The expression of vitellogenin (*Vg*) is regulated at the transcriptional level (Rhaikel *et al.*, 2004; Tufail and Takeda, 2008) by the hormones juvenile hormone (JH), ecdysone, and several other neuropeptides (Tufail and Takeda, 2008). According to Tufail and Takeda (2008) insects can be classified into one of three categories based on the mode of hormonal regulation of vitellogenesis used by the species: (a) type I includes insects that use only JH for *Vg* gene expression like Blattodea (Nagaba and Tufail, 2011), (b) type II includes insects that require JH and ecdysone like Diptera (Hagedorn *et al.*, 1975), (c) type III includes lepidopterans that require JH, ecdysteroids, and additional hormones to regulate their reproductive physiology like Lepidoptera (Wyatt and Davey, 1996; Rhaikel *et al.*, 2004).

Previous studies have implicated the ecdysone derivative, 20E as being the main regulator of vitellogenesis in mosquitoes (Spielman *et al.*, 1971; Hagedorn, 1985), however, the observation that haemolymph titres of 20E begin to increase *after* the start of yolk protein precursors (YPP) secretion (Racioppi *et al.*, 1986) suggests that this may not be true. This position is supported by another component of Redfern's (1982) work, which involved the injection of large, non-physiological doses of 20E into decapitated female mosquitoes. In his experiment, 20E induced only low levels of YPP expression. These reports hint that another stimulus is responsible for activating vitellogenesis.

Support for this notion comes from two similar reports in which *in vitro* application of physiological levels of 20E to fat body preparations from mature non-blood fed females stimulated vitellogenesis. Although this may appear contradictory to previous findings it is important to emphasize that the fat body preparations were cultured in amino acid rich media. (Deitsch *et al.*, 1995; Raikhel *et al.*, 1997). The fact that fat bodies are responsive to 20E in amino acid rich media suggests that they may act as a signal to the fat body, allowing 20E to activate YPP genes and the process of vitellogenesis. Uchida *et al.* (1998) demonstrated the same phenomenon using a relatively less invasive assay in which solutions containing amino acids and physiological levels of 20E were infused into the haemolymph of pre-vitellogenic females of the anautogenous mosquito, *Culex. pipiens* pallens. Most of the females (~70%) were able to complete egg development following this treatment and researchers noted that the increase in their amino acid concentration was similar to that of their blood fed counterparts. These data support the hypothesis that amino acids play a regulatory role in vitellogenesis but also lead to another important question: Which amino acids are essential for initiating vitellogenesis?

2.1.2 Amino acid regulation of vitellogenesis

The amino acids essential for alleviating the vitellogenic state of arrest are: leucine, isoleucine, lysine, phenylalanine, threonine, tryptophan, valine, cysteine, and arginine (Dimond *et al.*, 1956). Female *Ae. aegypti* fed artificial blood meals from which these individual amino acids were omitted failed to complete egg development. This suggested that all of the amino acids listed were regulators of egg development (Dimond *et al.*, 1956). Even more recently an amino acid infusion-based assay using a Culex model, showed the elimination of any one of these amino acids (plus asparagine) resulted in arrested egg development (Uchida *et al.*, 1998). These findings also coincide with another experiment conducted by the same group involving seven other species of anautogenous mosquitoes (Uchida *et al.*, 2001). Five of the seven species underwent successful egg development. Amino acid infusions failed to induce vitellogenesis in *Cx. halifaxii* and *Ae. japonicus,* suggesting that in some species the amino acid "trigger" is insufficient to promote complete egg development. Although the mode of action of these amino acids remains unknown it is widely accepted that some blood meal dependent signal induces the hormonal activation of vitellogenic genes (Figure 1.1; Attrado *et al.*, 2005).

2.1.3 Mating

In many insects, mating facilitates the transfer of male accessory gland constituents via the seminal fluid which induces a wide variety of behavioural and physiological changes in females. There is a growing body of evidence demonstrating their effects in female mosquitoes and other Diptera (Bloch Qazi *et al*, 2003; Dottorini *et al.*, 2007; Sirot *et al.*, 2008). Some of these constituents are proteinaceous and are called accessory gland proteins.

2.1.4 Accessory gland proteins (AGPs)

Some AGPs have been shown to act locally within the female reproductive tract, altering its morphology to influence sperm storage, sperm management and the outcome of sperm competition (Bloch Qazi *et al.* 2003; Wolfner, 2009). Still others are absorbed into the haemolymph and act indirectly through neural or endocrine targets (Monsma *et al.* 1990; Bloch Qazi *et al.* 2003; Wolfner, 2009) to increase feeding rate and host-seeking behaviour (Klowden and Lea, 1979; Carvalho *et al.* 2006), increase circadian flight activity (Jones and Gubbins, 1979), decrease female sexual receptivity (Shutt *et al.*, 2010), stimulate oogenesis and even increase production of immune related peptides (Soller *et al.* 1997; Peng *et al.* 2005) to enhance females' immune response to potential infections. Given the fact that mating and fertilization are decoupled in most insects, including mosquitoes, the transfer of AGPs during mating allows the male to enhance his direct fitness by increasing the reproductive investment of his mate (e.g., increasing time spent foraging). It has even been suggested that these proteins may enhance the mating male's fitness at the expense of the female's survivorship. Klowden and Chambers (1991) demonstrated that starved *Ae. aegypti* females were more likely to develop eggs after mating than were their non-mated counterparts.

2.1.5 Steroid hormones

In addition to AGPs the accessory glands of mosquitoes also produce steroid hormones. The vitellogenic hormone, JH involved in ovarian follicle development is synthesized and stored in accessory glands of male *Ae aegypti*. Recently, Pondeville *et al.* (2008) showed that *Ae. gambiae* males produce large amounts of 20E, previously described as the female sex steroid for mosquitoes and other insects. They demonstrated that the production of 20E in *An. gambiae* males is restricted to its reproductive accessory glands, as no 20E was detected in testes or other body parts of the insect. Males of *An. gambiae* produce 20E from the beginning

of their adult life and *in vivo* titres peak between 1-3 days post emergence. This coincides with the time required for males to reach sexual maturity before a first successful mating can take place. But perhaps the most interesting finding was that in *An gambiae,* males transferred high quantities of this steroid hormone to females during mating. Surprisingly, the stimulating capacity of male-derived 20E exceeds even that of vitellogenic ovaries, suggesting that vitellogenesis in this species may also be controlled by an exogenous regulatory mechanism. However this is true only of males that have reached sexual maturity (i.e., 5 days post emergence in *An. gambiae*, Huho *et al.*, 2006).

In previous studies, sexual maturity was scored by noting morphological changes in the accessory glands themselves (Mahmood and Reisen, 1982; Huho *et al.*, 2006). Mahmood and Reisen (1982) demonstrated that in *An. stephensi* the morphology of the male accessory glands and the quantity of their contents increases with age. Additionally, a peak in the degree of accessory gland repleteness, which is indicative of secretory material accumulation, is observed between 5 and 7 days post emergence (Mahmood and Reisen, 1982).

1.6 Study model

An. stephensi (Diptera: Culicidae) is a major malaria vector in many parts of Central Asia (Figure 1.2) and is used as a model in the present study based on its large medical and public health relevance, the ease with which it can be reared in the laboratory, and the availability of sequence information for this species (Nirmala *et al.*, 2006). *An.stephensi* is a holometabolous insect and undergoes four different life stages: egg, larval, pupal and adult. Female mosquitoes lay their eggs in aquatic habititats that range from small shallow puddles and man-made containers to sheltered lakes and marshes (Foster, 1995; Benedict, 2007). Larvae hatch from their eggs approximately 1-2 days after oviposition and feed on plant and animal matter suspended near the surface of the water (Benedict, 2007). Conditions in the larval habitat, including temperature, humidity, larval density, presence of conspecifics and predators, and food availability are factors that to the duration of the larval stages and adult body size and nutritional status at the time of emergence (Benedict, 2007). Larval diet is the largest contributor of adult size and health. Diet restriction at the immature stages delays the onset of pupation and produces smaller adults with fewer energy reserves (Benedict, 2007). As adults, males feed exclusively on sugars while females feed on both sugar and blood. Females exhibit obligate anautogeny (Nirmala *et al.*, 2006).

An. stephensi can store nutrients derived from sugar and/or blood as sugar, glycogen, protein and lipid in flight muscles and the abdominal fat body. Lipids are also stored in the fat body and are essential for oogenesis in females (Foster 1995).

1.7 Study objective

Considering the importance of sugar feeding behaviour on the life history of male mosquitoes and some of exogenous control mechanisms regulating female vitellogenesis, the present study was conducted to test the hypotheses that males under varying diet regimes will elicit varying vitellogenic responses in their mates following mating. The level of vitellogenin expression in the mates of nectar- or honeydew-fed males was compared using a real-time quantitative RT-PCR assay.

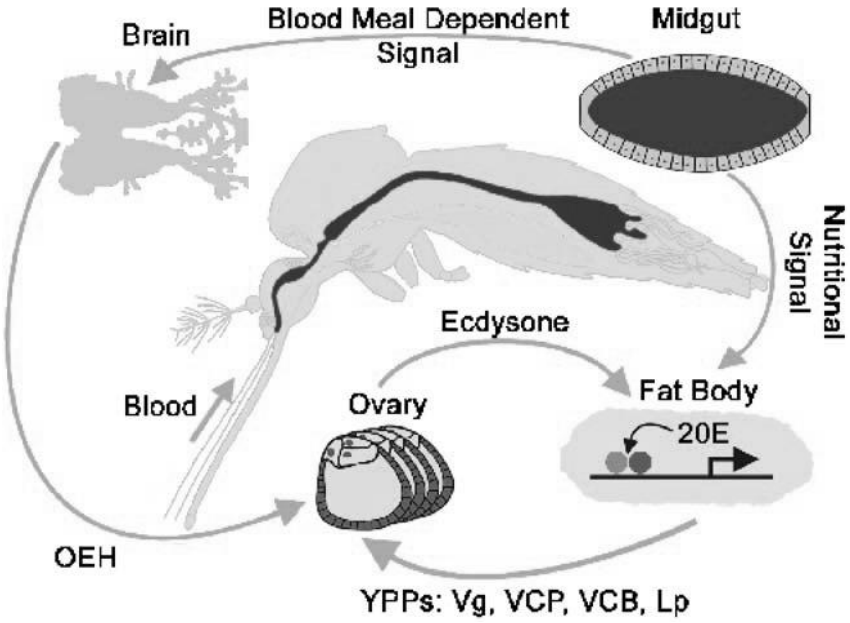

Figure 1.1. Schematic diagram illustrating the blood meal activation of vitellogenesis in anautogenous mosquito. The acquisition of a blood meal by the mosquito results in the release of a blood meal dependent-signal from the midgut to the brain. In response to this signal, the brain releases the peptide hormone OEH, which stimulates the ovaries to synthesize and release ecdysone. The ecdysone travels to the fat body and is converted to 20-hydroxyecdysone, which activates yolk protein precursor gene expression.Yolk proteins are then synthesized in the fat body, secreted, and transported to the ovary for incorporation into the developing oocytes. OEH: Ovarian ecdysiotropic hormone; 20E: 20-hydroxyecdysone; YPP: yolk protein precursor; *Vg*: vitellogenin; VCP: Vitellogenic Carboxypeptidase; VCB: Vitellogenic cathepsin B; Lp: Lipophorin. (Retrieved from Attardo *et al.* 2005)

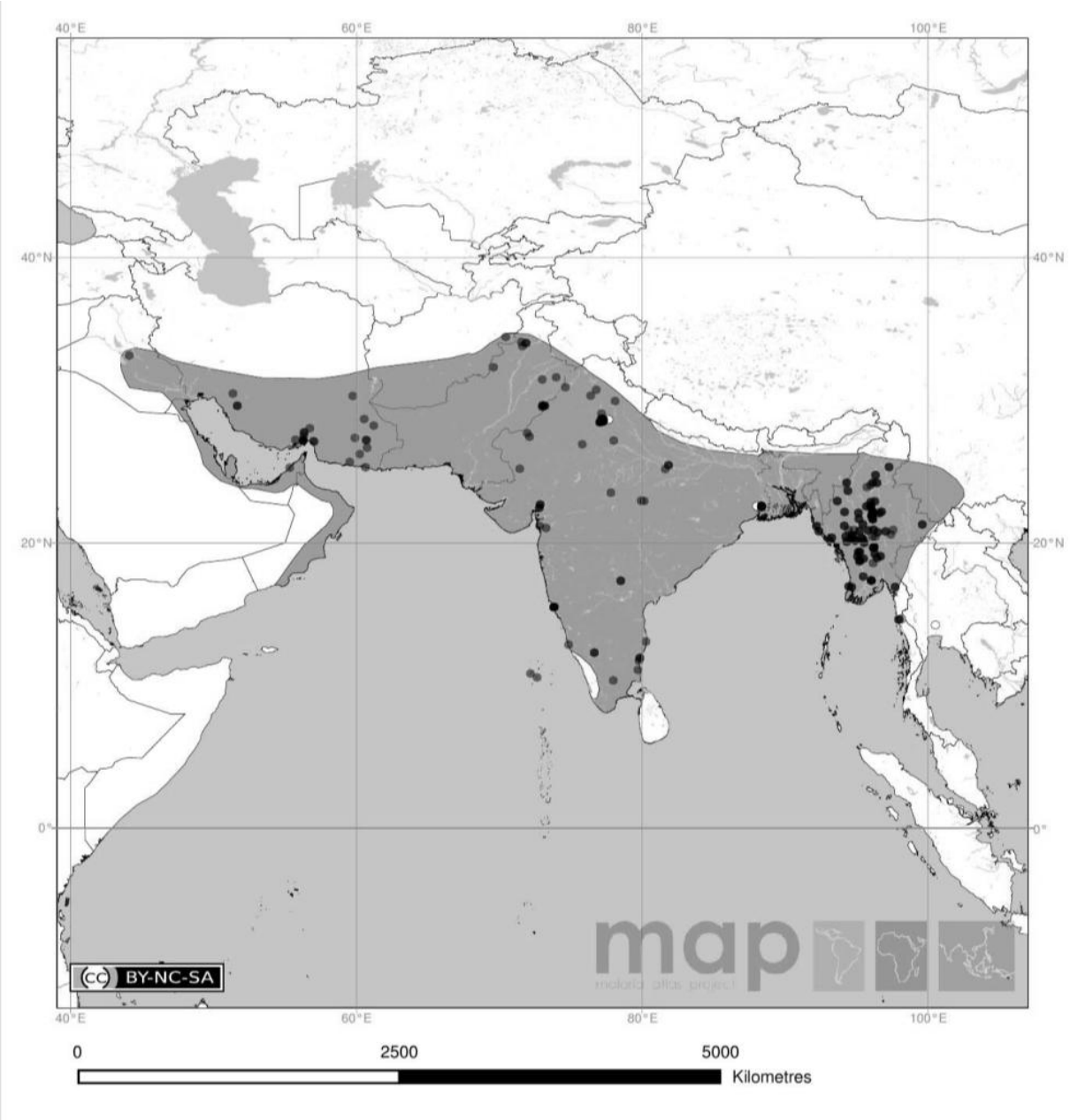

Figure 1.2. Global distribution of *Anopheles stephensi* Liston model used in present study. In grey are the hypothesised ranges which encompasses expert opinion and the species occurrence records collected from the searches of the formally published literature (Retrieved from Hay *et al.*, 2009)

Figure 1.3. (a) Male *Anopheles stephensi*. Males are easily distinguished by their feathery or plumose antennae. (b) Female *Anopheles stephensi*

Figure 1.4. Dissected male *Anopheles stephensi* terminalia. The accessory glands (AG) are visible as the faint yellow sacs near the terminalia.

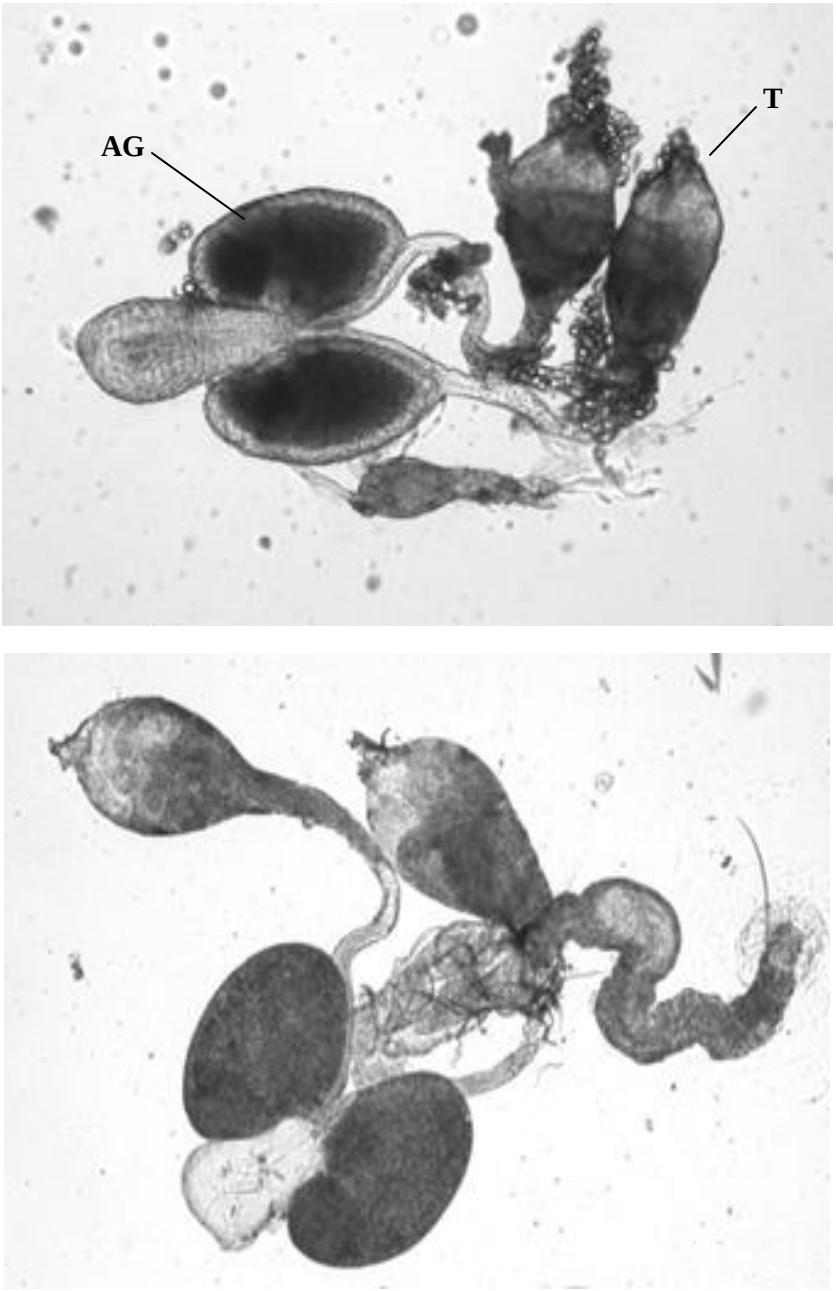

Figure 1.5. Accessory glands (AG) and testis (T) of a 3 day old *Anopheles gambiae* mosquito (ovoid structures with translucent perimeter, *top*). Accessory glands and testis of a 5 day old *Anopheles gambiae* mosquito (dark ovoid structures, *bottom*) (Retrieved from Huho *et al.*, 2006)

2.2 Methods

2.2.1 Mosquito rearing

Anopeheles stephensi were maintained in colony in an incubator set at 27^{o}C, 70% relative humidity and 12L: 12D photoperiod. Males and females were housed together in breeding cages measuring 30 cm^{3} (Figure 1.6) and offered a diet of 10% sucrose solution (as recommended in Benedict, 2007, and references there in). Mosquitoes were offered sheep's blood every 3-4 days using a glass feeder (as described in Kasap *et al.*, 2003). Oviposition trays with moistened filter paper were placed with caged adults 24 hour following each blood meal, and eggs were collected the following day. Larvae were hatched into Petri dishes and were fed a standard diet (Laguna^{TM} Goldfish and Koi Chow, Rolf C. Hager (USA) Corp. Lansfield, MA, finely ground powder). Larval density was maintained at 100-120 individuals per dish. For all experiments, pupae were separated into individual 20 mL scintillation vials with 8-10 mL of water and allowed to emerge into a separate breeding cage. Emerged adults were sexed and housed in same-sex cages containing either 10% (w/v) sucrose solution, artificial nectar solution or artificial honeydew solution for the first 5 days following their emergence.

Figure 1.6. Adult mosquito housing containers. Collapsible breeding cage (BioQuip, CA 90220) used to house stock *Anopheles stephensi* adults (left). Modified ice-cream tub with netted cap used to house test subjects (right).

2.2.2 Diet composition

The artificial sugar diets used in all the experiments were intended to simulate naturally occurring nectar and homopteran honeydew. The artificial nectar (AN) diet was composed of 4.0% (w/v) fructose, 4.0% (w/v) glucose, and 2.0% (w/v) sucrose. The artificial honeydew (AH) diet was composed of 6.0% (w/v) fructose, 4.0% (w/v) glucose, 2.0% (w/v) sucrose, 8.0% (w/v) melezitose, 0.1% (w/v) D-asparagine, and 0.1% (w/v) D-glutamine. All diets were prepared separately in sterile conical flasks, stored in a refrigerator and presented to the test animals on a loosened dental wick protruding from the top of 10 mL glass shell vial. The amino acids, D-asparagine and D-glutamine have also been found in certain honeydew secretions (Blüthgen *et al.*, 2004). The ingredients of both artificial diets were developed based on previous studies in which chromatographic techniques were used to analyze the sugar composition of naturally occurring nectars (floral and extrafloral nectars) and homopteran honeydew secretions (Auclair, 1963; Van Handel *et al.*, 1972; Hussain *et al.*, 1974; Tarczynski *et al.*, 1992; Barnes *et al.*, 1995).

2.2.3 Mating experiment

Pupae were collected from the stock colony over a 48 hour period and allowed to emerge in a new breeding cage. Adults were sexed within the first 8-10 hours and males were transferred into separate containers for 5 days post-emergence. Each container contained one of three different sugar meals (artificial nectar, artificial honeydew and 10% sucrose) which were replenished daily. Females were split into two groups and housed in breeding cages (to facilitate blood feeding) for 5 days post-emergence. One group was presented with 10% sucrose diet and a single blood meal at 4 days post-emergence, while the other group was presented with only the 10% sucrose diet (a staple diet in laboratory colonies, Benedict, 2007).

At 5 days post-emergence females were presented with an excess of mates with different nutritional histories. The various mating arrangements used during the mating experiment are illustrated in Figure 1.7.

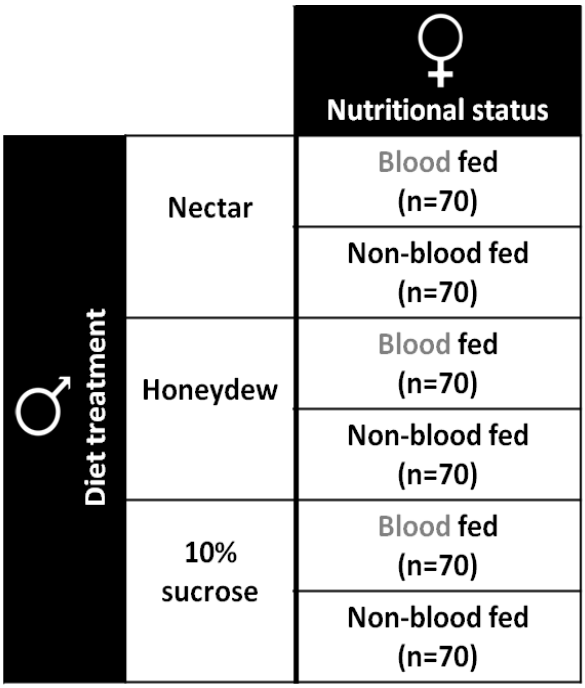

Figure 1.7. Mating experiment design. Males were provided with one of three diets: artificial nectar, artificial honeydew and 10% sucrose for the first 5 days post-emergence. Blood fed females were provided with a single blood meal at 4 days post-emergence and 10% sucrose diet *ad libitum*, while non-blood fed females were provided only the 10% sucrose solution. All sugar solutions were replenished daily until the end of the experiment. At 5 days post-emergence females were presented with an excess of males for 8 to 10 hours to allow mating.

Males were aged to 5 days prior to mate presentation so that: (1) the accessory glands of *An. stephensi* would become fully mature (Mahmood and Reisen *et al.*, 1982) and (2) females would exhibit increased mate receptivity (Reisen *et al.*, 1979). Virgin blood fed females (n=60) and virgin non-blood fed females (n=60) were also transferred to two separate containers and would later serve as controls for the effect of mating and blood feeding, respectively. Females were presented with an excess of males for 8 to 10 hours, after which all the males were removed from the breeding cages. Twenty-four hours after the start of mating period 14-20 females were removed from each housing container for dissection. This procedure was repeated with another group of 14-20 females at 36h, 48h and 60h after the start of mating period.

2.2.4 Dissection protocol and confirmation of insemination status

Females were first aspirated and killed by placement in -20^{o}C freezer for 10 minutes. Under a microscope (Leica MS5, Leica Microsystems) each female was then dissected on a glass slide in a drop of 1X phosphate buffered saline (PBS). To ensure that the entire fat body was excised the anterior most incision was made between the mid- and hind- legs while the posterior most incision was made right between the 4th and 5th abdominal segments. Each abdominal fat body preparation was then transferred using sterile forceps to a labelled 1.5 mL Eppendorf tube containing 50 µL of RNA stabilization reagent (Qiagen) and stored at -20^{o}C until use. The remainder of the abdomen of each mated female was stored in labelled 0.6 mL Eppendorf tube with a drop of 1X PBS and temporarily stored at 4^{o}C. This was done to confirm the females' insemination status at the time of dissection. The last abdominal segment houses the spermathecae which were inspected for presence of sperm under a compound microscope (Alphaphot-2 YS2, Nikon) following the technique described by Benedict (2007). Only inseminated females were used in subsequent gene expression analysis.

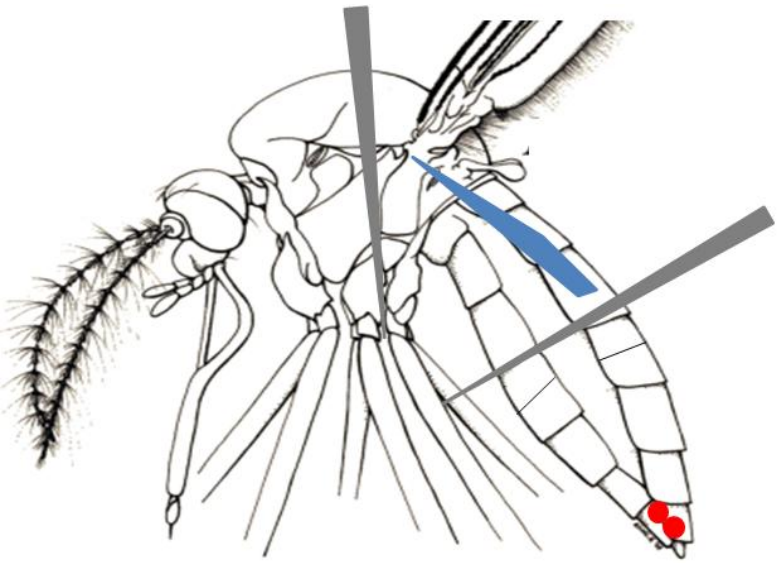

Figure 1.8. Schematic illustration of the dissection protocol used during the excisions of females' fat body (blue). Also shown are the sperm storage organs or spermathecae (red) which were examined under a microscope to confirm females' insemination status. The number of spermatheca varies among the culiciforms (e.g. 1 in *Anopheles*, 2-3 in *Aedes* and 3 in *Culicids*, Yuval, 2006).

Figure 1.9. Dissected female *Anopheles stephensi* terminalia. Inspection of the sperm storage organ or spermatheca (SP) of mosquitoes is commonly used to assess mated status of the female.

Figure 1.10. Mechanically ruptured spermatheca. Absence of sperm near the rupture site was indicative of non-mated female.

2.2.5 RNA extraction and RT-PCR assay

To determine the expression patterns of the vitellogenin gene in the mates of the experimental and control males, real time PCR was conducted using RNA from female abdominal fat body preparations. Total RNA was isolated using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions for specimens stored in RNA stabilization reagent. A volume of 2 µL of total RNA was used to synthesize first-strand cDNA using Superscript III Reverse Transcriptase (Invitrogen). Reverse transcription was carried out using oligo-$(dT)_{20}$ primer (Invitrogen) in a 20 µL reaction volume at 50^{o}C for 50 m followed by termination of the reaction at 70^{o}C for 15 m. All cDNA synthesis reactions were carried out immediately after RNA extraction and to minimize RNA degradation and potential variation in reverse transcriptase efficiency. Real time PCR was performed on first-strand cDNA using custom primers against *An. stephensi vitellogenin gene* 1 (*AsVg1*, complete sequence deposited in GenBank by Nirmala *et al.*, 2006). All optimized gene-specific sense and antisense oligonucleotide primers and TaqMan probes (Applied Biosystems) were designed using Beacon Designer software (Premier Biosoft International, CA). Gene-specific primers against the *AsVg1* gene were used to amplify a 260 bp product (Table1). As an internal control, the transcript of the *An. stephensi ribosomal protein S6* gene (*AsRPS6*) was amplified using the custom primers to yield a 233 bp product (Table 1).

Real-time qPCR was carried out in a MyiQ Thermal Cycler (BioRad, CA) according to the manufactures instructions in a 96-well microtiter plate with a 30 µL reaction volume. Each reaction contained 5 µL of cDNA template, 2.3 µL of 10X Standard Taq reaction buffer, 2.6 µL of 25mM Mg^{2+}, 0.3 µL of 10 µM dNTP, 1.15 µL of primer-probe mixture, 0.3 µL of 5U/µL Taq DNA Polymerase (Norgen) and sterile H_2O to a volume of 25 µL. A negative control (without cDNA template) and a no-RT control (with total RNA template) were performed in each run. Reaction conditions were as follows: preincubation was performed for 2 m at 95°C to

denature the target DNA and activate Taq DNA Polymerase followed by amplification for 40 cycles of 15 s at 95°C and 1 m at 59°C. The cycle number at the threshold was used as the threshold cycle (C_t). Data were analyzed using the Livak method (a comparison of relative transcript abundance, described by Livak and Schmittgen, 2001) on a Microsoft Excel based spreadsheet (Microsoft, WA) and expressed as normalized expression ratios (NERs). The expression of *An. stephensi* vitellogenin 1 gene (*AsVg1*) in all test treatments was calibrated against the expression of *AsVg1* in blood fed virgin treatment, as blood is the primary stimulus for vitellogenesis.

2.2.6 Statistical analysis

Data were analysed for normality (using the Wilks-Shapiro test) and homogeneity of variances (using Levene's test). Differences in mean NERs among the various diet regimes for each time interval after the start of mating were analyzed by one-way ANOVA followed by a Tukey HSD pairwise comparisons test. In all instances, values of $p < 0.05$ were considered significant. Data sets that did not conform to normal distribution were analysed using non-parametric Kruskal-Wallis test. All statistical tests were carried out using Statistix 8 (Analytical Software, Florida) and IBM SPSS Statistics Version 20 (IBM Corporation, New York). Figures were plotted using Microsoft Excel (2010; Microsoft Corporation, Washington).

Table 1. List of primers used in RT-PCR protocol. Shown are the primer sequences, annealing temperatures (Ta), lengths of the corresponding PCR products and GenBank accession numbers of the nucleotide sequences. F, forward; *AsVg1*, *Anopheles stephensi* vitellogenin 1; *AsRPS6*, *An. stephensi* ribosomal protein S6; R, reverse. Primers were designed using Beacon Designer 7.0 (Premier Biosoft Int.)

cDNA	Forward and reverse primers	Ta(^{o}C)	Product	GenBank
AsVg1	F: 5'-ATACGACAAGGACTTCAT-3' R: 5'-CGTTGTAGTATCCAGAGT-3'	59.1	260	DQ442990
AsRPS6	F: 5'-CTCGGAGAAGGACAAG-3' R: 5'-CCTTCTTGCCATCCTT-3'	60.0	233	AY237124

2.3 Results

2.3.1 Mating experiment

At the end of the mating experiment, the insemination status of the blood fed (BF) and non-blood fed (NBF) females presented with nectar-fed, honeydew-fed, or 10% sucrose fed mates was confirmed (Table 2). Not included in Table 2 are the BF females (n=58) and NBF females (n=59) that were the unmated controls. Interestingly, females were more likely to be inseminated if they had previously blood fed (independent samples t-test: t= 4.88, df = 2, $p <$ 0.05). This trend was apparent across all mating treatments (Table 2). Females that had died prematurely or due to unnatural causes (e.g. immobilized in sugar soaked dental wicks) were not used in the downstream gene expression analysis.

2.3.2 Post-copulatory changes in *AsVg1* expression in blood fed *An. stephensi*

Using quantitative real time RT-PCR the time course of *Vg* expression was examined. Since the extracted RNA used in this experiment was not treated with DNAses to remove DNA, a no-RT control was included in every 96-well microtiter plate. All controls for DNA contamination (n=10) produced C_t values above 37 (data not shown); thus a sample C_t value of 35 was chosen as the cut-off for false-positives. It was found that *Vg* expression increased from 0 to 24h post-mating, peaked at around 36h post-mating then began to decline between 48 and 60h post-mating (Figure 1.11). The Wilks-Shapiro test confirmed that all data sets presented in Figure 1.11 were normally distributed ($p >0.05$) with the exception of NBF virgin group ($p <0.05$ at 24h and 48h post-mating). Results of a one-way ANOVA indicated differences ($F_{(3,47)} = 4.48$; $p <0.01$) in mean NERs of *AsVg1* among the test treatments at 36h post-mating. Tukey's *post-hoc* comparisons showed that the mean NER of *AsVg1* at 36h post-mating was 4-fold greater in the mates of nectar-fed males (mean NER = 4.24 ±0.67; $p<0.05$) than in their unmated BF counterparts (represented by dotted line, Figure 1.11). Mates of

honeydew- and 10% sucrose-fed males (mean NER =1.93 ±0.68 and 2.01±0.42, respectively) also exhibited elevated *AsVg1* titres relative to BF virgins, but interestingly, the level of expression was roughly half that of females mated to nectar-fed males. Figure 1.11 also shows the mean NER of NBF virgins was significantly lower than any other test group (mean NER = 0.08±0.01; $p < 0.05$). A similar trend is also evident at 48h post-mating ($F_{(3,39)} = 6.61$; $p < 0.05$) with mean NER of *AsVg1* being greater among females mated to nectar-fed males (mean NER = 3.90 ±0.56; $p < 0.05$). There was no difference in mean NER of *AsVg1* among the experimental diet treatments at 24h and 60h post-mating ($p > 0.05$, at both intervals).

2.3.3 Post-copulatory changes in *AsVg1* expression in non-blood fed *An. stephensi*

The time course of *AsVg1* expression was also investigated in females that did not receive a blood meal prior to mating (Figure 1.12). As in the treatments described above, the BF virgin group was used to calibrate the expression of *AsVg1* in all the categories presented in Figure 1.12 so that comparisons between BF and NBF females could be made. Only four of the sixteen data sets presented in Figure 1.11 conformed to a normal distribution ($p > 0.05$; Wilks-Shaprio test). Therefore, non-parametric Kruskal-Wallis test followed by Tukey's *post hoc* comparisons test was used to compare the mean NERs at each time interval. No difference (Kruskal–Wallis test, $p > 0.1$) in NER was detected between the different treatments.

Table 2. Summary of females' insemination status protocol.

Diet treatment of male mates	Percentage of BF females mated (n=)	Percentage of NBF females mated (n=)
Nectar	71.7% (53)	43.4% (53)
Honeydew	80.4% (56)	59.2% (49)
10% sucrose	87.3% (55)	44.7% (47)

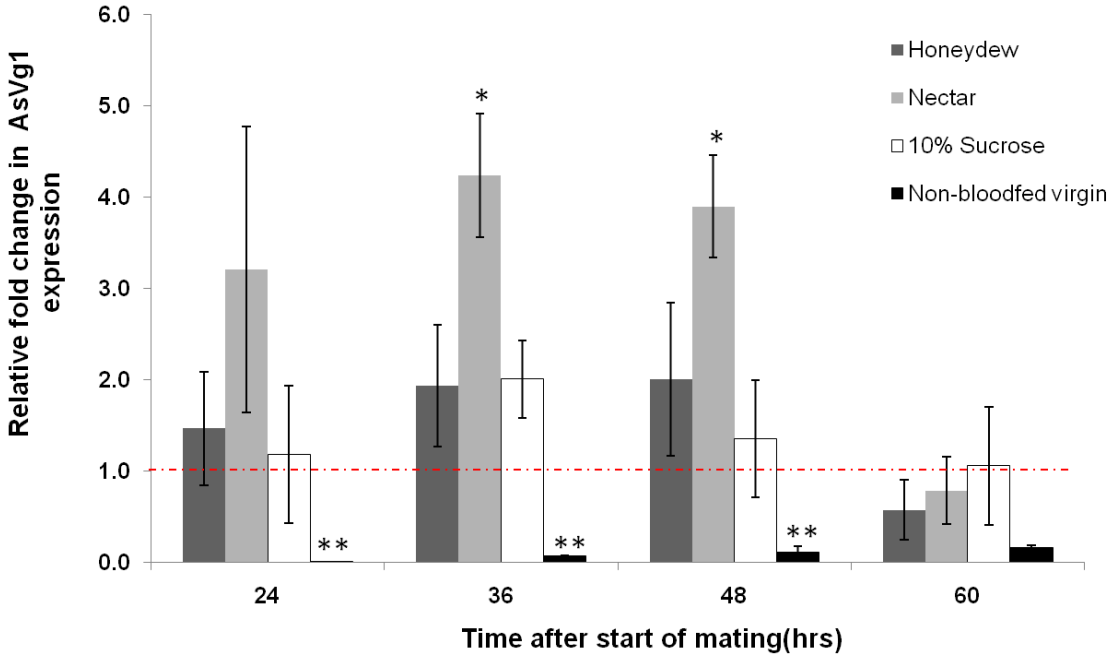

Figure 1.11. Post-copulatory changes in *AsVg1* expression in blood-fed (BF) *An. stephensi*. The figure shows the mean normalised expression ratio (NER) of *AsVg1* in BF female fat bodies (±S.E.M.). A peak in *AsVg1* mRNA titre was observed in the mates of nectar-fed males at 36h post-mating. The dotted line represents the NERs of blood fed female virgins against which all test treatments were calibrated. Asterisks (*) indicate treatments with mean NERs that were significantly different (ANOVA: $p<0.05$).

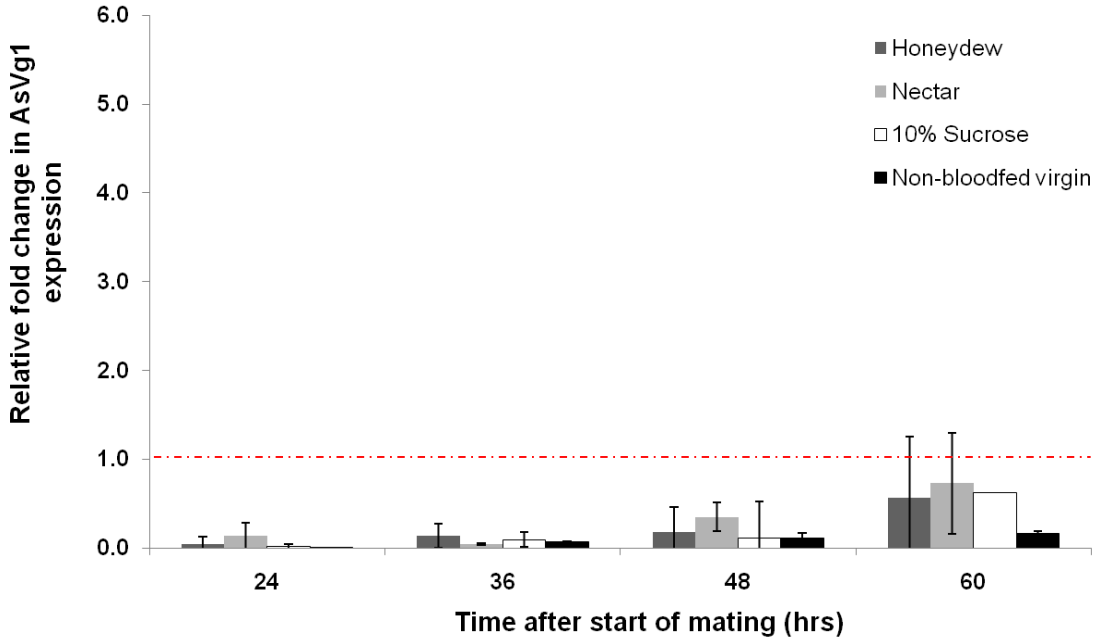

Figure 1.12. Post-copulatory changes in *AsVg1* expression in non-blood fed (NBF) *An. stephensi*. The figure shows the mean normalized expression ratios (NERs) of *AsVg1* in NBF female fat bodies (±S.E.M.). The dotted line represents the NER of blood fed female virgins against which all test treatments were calibrated. There was no difference in *AsVg1* expression at any time interval after the start of mating (Kruskal-Wallis test: $p>0.1$).

2.4 Discussion

In the present study, real-time quantitative RT-PCR was used to compare the changes in *Vg* expression in female *An. stephensi* induced by mating to males with different nutritional histories. Of the diet types examined, a higher fold-change in *AsVg1* mRNA (at least 2X at its peak, Figure 1.11) was detected in the mates of nectar-fed males ($p<0.05$; Figure 1.11) than in mates of honeydew-fed males. This difference in *Vg* expression persisted until 48h post-mating, after which the expression level began to decline in all treatments. These findings are consistent with the report of Klowden and Chambers (1991) which suggested that sucrose-deprived males were less likely to stimulate oogenesis in sucrose-deprived blood-fed females than if the males were maintained on sucrose before mating. That nectar-fed males in the present study were able to elicit a relatively greater vitellogenic response in their mates, has significant implications on our understanding of the role of sugar feeding on male mating competence and female fecundity.

In mosquitoes, especially anautogenous species like *An. stephensi*, vitellogenesis is an extremely critical physiological event since its initiation is blood-meal dependent. It follows then that there must be physiological coping mechanisms in place to deal with the conflicting demands of survival and reproductive effort when blood is a limiting resource. One of these mechanisms is pre-vitellogenic oocyte arrest in which poor larval nutrition results in adults with stunted pre-vitellogenic ovarian follicle development. This condition is alleviated by the acquisition of two blood meals – the first to develop ovarian follicles to the normal pre-vitellogenic state and the second to initiate active oogenesis (Attardo *et al.*, 2005).

The time course of vitellogenin upregualtion reported here gains support from Sappington *et al.* (1995) who reported a similar trend in vitellogenin production in blood fed *Aedes aegypti*. The quantity of *Vg* increased rapidly between 8 and 24 hours post-blood feeding and gradually declined between 24 and 36 hours. However, it should be noted that the females

did received only a blood stimulus in that study. It is possible that vitellogenic response of females observed in this study may have been the result of the blood stimulus and mating acting synergistically.

In the wild, a preference for nectar feeding may grant males selective advantages over their honeydew feeding counterparts by allowing them to manipulate the reproductive output of nutritionally stressed females, making them more fecund despite their limited reserves.

When the effect of mating on *Vg* expression was investigated in the absence of a blood stimulus (Figure 1.12), no differences in *AsVg1* mRNA were detected among all mating treatments. It is likely that mating alone failed to elicit a vitellogenic response because *An. stephensi* is an obligatorily anautogenous species; thus blood-meal deprivation of the females in this experiment ensured that vitellogenesis was maintained in a state of arrest. However, it is worth noting that there was a trend toward increasing *AsVg1* expression as time elapsed (Figure 1.12) but the expression level did not exceed that of mated females (indicated by red dotted line, Figure 1.12).

Obligatorily anautogenous mosquitoes generally cannot undergo egg development without a blood meal. However, some species have autogenous strains or may exhibit plasticity under selective pressure. One such species is *Ae. aegypti*, which is usually an obligatorily anautogenous mosquito that feeds exclusively on humans. In 1977, Trpis identified an autogenous strain of *Ae. aegypti* in East Africa that lives in the jungle isolated from its human host. It is thought that these populations adopted autogeny as an alternate reproductive strategy in response to a reduction in host density (Trpis, 1977). Autogeny has also been previously induced in lab-reared colonies of *Ae. aegypti* by supplementing the larval diet with extra protein and restricting adult blood feeding (Lea, 1964). Since the strain of *An. stephensi* used in the present study was anautogenous, it is likely that the *Vg* expression pattern observed in the mated NBF females was an artifact of the larval rearing conditions.

Interestingly, the pattern of *Vg* expression in the mates of 10% sucrose fed males and honeydew-fed males was very similar, despite differences in the composition of these diets. The concentration of sugar in the honeydew solution used in the mating experiment was twice that of the 10% sucrose solution and the nectar solution. However, given the fact that all sugar meals were provided *ad libitum* for 5 days prior to mate presentation, it is unlikely that the observed differences in *Vg* expression induced by mating were caused by varying degrees of caloric deficiency of the males. Furthermore, mosquito saliva contains the enzyme melezitase (Foster, 1995), which hydrolyses melezitose. Even though the digestion of melezitose may be energetically demanding (as was demonstrated in *Ae. albopictus*; Burkett *et al.*, 1998), mosquitoes do possess the enzymatic ability to digest it. Thus the possibility of any deleterious effects induced by its accumulation in the midgut of the mating males can be ignored. The differences in female *Vg* expression observed in this study must be due to quantitative or qualitative differences in the seminal fluid transferred during mating.

2.5 Summary

Reproduction in general is an important target for strategies designed to control the population sizes of disease-vectoring insects, including anopheline mosquitoes. Today most control strategies developed target female reproduction directly (e.g. mosquito repellent lotions, nets and carbon dioxide expelling traps). This study demonstrates the importance of male nutritional ecology on post-copulatory physiological changes in females. However, a detailed understanding of the molecular basis of the vitellogenic response observed here will be essential to further our understanding on how to population dynamics of wild populations are influenced by male nutritional history. Since males do not ingest blood, their survival, flight activity, and mating capacity is completely dependent on reserves carried over from larval feeding and from adult sugar feeding. The question remains then: Would the vitellogenic responses ultimately lead to variation in male reproductive success (e.g. increased egg output)? More specifically, what are the direct or indirect benefits to overall fitness that result from varying nutritional histories?

To address this question fully it is necessary to turn to facultatively autogenous models in which the female egg production can be initiated by mating alone. It is important to note, that the benefits (or costs, if any) associated with a preference for a given sugar source may not necessarily be reflected female egg output.

CHAPTER 3

Correlates between male diet composition and female life history traits in the autogenous mosquito, *Culex molestus* (Diptera: Culicidae)

3.1 Introduction

Mating is one of the most important behaviours that characterize the life history of mosquitoes, and yet the nutritional and environmental factors which regulate it remain largely understudied. Mosquitoes depend on sexual reproduction for species maintenance; therefore this aspect of mosquito biology should receive the highest attention when seeking new avenues for mosquito control and interventions for mosquito-borne disease. This review will highlight some of the key components of mating (specifically, the role of males) and highlight some of the intrinsic and extrinsic factors that regulate each component. For male mosquitoes, reproductive success typically hinges on three main processes: copulation, insemination and fertilization.

3.1.1 Copulation success

In order to copulate, males must first engage in swarming behaviour. The basic mating system of the Culicidae, and most Diptera that undergo an aquatic immature stage followed by synchronized emergence, is the polyandrous swarm (Yuval, 2006). These consist mainly of males maintaining flight over the swarm site and as a result exhibit heavily male-skewed sex ratios. The swarm sites of different species are segregated temporally and/or spatially (Shuster and Wade 2003; Yuval, 2006).

Field studies of several anopheline species indicate that swarm sites are typically over or near aquatic environments, with swarming activity peaking around twilight hours. Among these are the malaria vectors *Anopheles gambiae* (Charlwood and Jones, 1980), *An. funestus* (Charlwood *et al.* 2003), *An. culicifacies* (Reisen *et al.* 1982), *An. stephensi*, and *An. subpictus* (Panicker and Rajagopalan, 1984), *An. franciscanus* (Belkin *et al.* 1951), and *An. freeborni* (Yuval *et al.* 1993). However, exceptions to the polyandrous mating swarm have been reported suggesting that swarming is not necessarily a prerequisite for successful mating. Lounibos *et al.* (1998) found that mating in some neotropical species like *An. darlingi* can occur in the absence of swarms, as female insemination was observed outside houses (possibly near blood hosts). Many laboratory strains of culicine and aedine species also exhibit swarming behaviour (Reisen *et al.*, 1985; Yuval, 2006). Insemination rates of laboratory strains of culicines suggest that, at least in some species, mating can occur in confined spaces that are not conducive to swarm formation. This ability to mate in confined spaces is described as stenogamy and has been reported in many *Culex* species, including *Cx. tarsalis* (Reisen *et al.*, 1985) and *Cx. molestus* (Kassim *et al.*, 2012a).

Researchers have also reported male swarming in the immediate vicinity of hosts among members of the *Ochlerotatus* genus. Most anopheline and culicine males are incapable of detecting host cues (McIver *et al.*, 1980); however some aedine (e.g. *Aedes. aegypti*; described in Yuval, 2006) and mansonian (e.g. *Mansonia uniformis*; McIver *et al.*, 1980) males have been shown to swarm near vertebrate hosts, where they most likely benefit from high female encounter rates. Swarming mosquito species tend to breed quite rapidly and are capable of establishing viable populations with high dispersion rates from low volume aquatic environments (Yuval, 1994; Takken *et al.*, 2005).

Regardless of the species specific nature of swarm markers, sexually receptive females that enter swarms are detected by their lower wing beat frequency (Howell and Knols, 2009).

Males become competent to mating after their external genitalia undergo a morphological change which is characterized by inversion of the terminalia. This generally occurs within the first 12 and 24 hours following emergence (Takken *et al.*, 2005; Howell, 2009). Courtship is difficult to discern given the fact that mating on the wing lasts 10 to 30 seconds. Sexually mature males are characterized by erect antennal fibrillae, which allow them to recognize the wing beat frequency of females attending swarms (Takken *et al.*, 2005). Brogdon (1994) found that the differences in wing beat frequencies between closely related species were distinct enough to serve as a reproductive barrier. However, there is considerable overlap between amplitudes of the wing beat frequency among members of the *An. gambiae* complex and postulated that additional mechanisms must be at work to facilitate conspecific recognition prior to mating (Tripet *et al.*, 2004).

Considering the duration of females' swarm attendance, the lack of discernible courtship and the heavily male-skewed sex ratio, female mate choice presumably occurs indirectly (e.g., high egg hatch rate) depending upon which individual male they mate (Andersson, 1994). Another consequence of the polyandrous mating system is a disproportionate variance in copulation success among the sexes. A male-baised sex ratio ensures that females will most likely find a mate, while intrasexual competition reduces the likelihood of males doing so (Reisen *et al.*, 1981; Shuster and Wade, 2003).

The underlying factors that determine male copulation success have not been extensively studied. One study by Yuval *et al.* (1993) found that copulating male *An. freeborni* were significantly larger than swarming males. While adult size at emergence was positively correlated with greater mating success in *An. freeborni*, this was not the case in *An. gambiae* and *An. funestus* (Charlwood *et al.*, 2002). This suggests that the effect of adult size may be species specific. In any case, given the duration of time spent attending swarm sites and the fact

larval reserves can only sustain adults for a few days following emergence (Foster, 1995), copulation success for swarming males must ultimately depend on sugar feeding.

3.1.2 Insemination

This aspect of mosquito biology presents some interesting avenues for optimizing male reproductive success, especially considering the individual male's low probability of locating a mate. Given this low probability, the reproductive investment of a male that manages to copulate is secured by assuring his mate will not copulate again. Once mated anopheline and culicine females become refractory to further mating (reviewed in Baldini *et al.*, 2012). This refractory response is mediated in part by the presence of sperm and AGPs in the spermathecae (Klowden, 2001). In some anopheline mosquitoes, seminal fluid secretions actually coagulate into a gelatinous plug that is deposited in the female atrium and acts as a barrier against further copulations. This barrier lasts only between 24 and 48 hours (Giglioli and Mason, 1966), but is sufficiently long enough to ensure the paternity of the subsequent batch of eggs. AGPs have also been implicated in some of the long term barriers to remating (e.g. induction of monogamy in Shutt *et al.*, 2010; Baldini *et al.*, 2012). In some aedines, peptides produced by the accessory glands target the female nervous system and reduce female sexual receptivity (Helinski *et al.*, 2012). This refractory behaviour was shown to be quite long-lasting as females injected with a low dose of seminal fluid proteins were not inseminated when exposed to males up to 34 days post-injection (Helinski *et al.*, 2012). Despite the increasing body of information regarding the post-mating response induced by these AGPs, the environmental factors that regulate them remain largely unknown.

Male age is one factor that has been implicated. Mahmood and Reisen (1982) found that male *An. stephensi* do not reach their peak reproductive potential until they are at least 5 days old. This is typically determined by examination of the accessory glands which become replete

as males age. In some strains of *An. gambiae* and *An. arabiensis* optimal mating occurs with 5 to 7 day old males (Takken, 2005), possibly coinciding with the maximal level of accessory gland repletion in those species. When considering the implications of these laboratory studies however, it is important to remember that unnaturally high mate encounter rates may promote mating far earlier than would be expected in the field. Such was the case in the aforementioned study, with peak insemination rate observed at 3 days post-emergence (Mahmood and Reisen, 1982). Although these studies have generated an important body of knowledge on the age effect on female insemination rate in the laboratory, the effects of age and other environmental factors (e.g. sugar host availability) on insemination rate in the field have yet to be investigated. The importance of sugar feeding on insemination rate is highlighted in the experiment of Stone *et al.* (2009). Sugar-fed males were able to inseminate 5 times as many females as sugar deprived controls. This may have been due to an inability to accumulate seminal fluids and/or sperm on larval reserves alone. Coupled with the fact that larval reserves cannot sustain males until their accessory glands mature (>3 days, Mahmood and Reisen, 1982; Benedict, 2007 and references therein) it is safe to assume that sugar feeding must play a significant role in successful insemination.

3.1.3 Fertilization

Sperm capacity of male mosquitoes is directly proportional to fertilization potential (Ponlawat and Harrington, 2007). It follows then that the factors that increase a male's sperm capacity (e.g. age and body size) can be used as predictors of a male's ability to fertilize eggs. Given that females can acquire and store sufficient sperm from their first mate to fertilize a lifetime supply of eggs (Rogers *et al.*, 2008), the entire reproductive output of a female mosquito can potentially depend on a single successful insemination and remating may not be required at all. Yuval (2006) suggested that the presence of multiple spermathecae in some

mosquitoes (e.g. 3 spermathecae in *Culex*) may allow females to segregate and discriminate between ejaculates of successive mates. However, field studies show that remating is very unlikely in wild populations. Tripet *et al.* (2003) using DNA-analysis of sperm in the spermathecae of field-caught *An. gambiae,* found that only 6 in 239 females had mated multiply. Thus, in mosquitoes the role of spermathecae as instruments of cryptic female choice may be limited to the laboratory settings, where remating is more likely to occur.

That age can influence sperm capacity gains support from the work of Mahmood *et al.* (1982; 1986). The number of spermatocysts decreased and the length of the sperm reservoir increased with age in *An. stephensi* (Mahmood and Reisen, 1982) and *Cx. tritaeniorhyncus* Giles (Mahmood *et al.* 1986). Another predictor of mating success and fitness in insects is body size. The upper limit of body size in mosquitoes is set by larval food availability and larval density. Adults that are larger at emergence will correspondingly possess larger reproductive organs and a greater total number of gametes than smaller individuals.

Ponlawat and Harrington (2007) found that large *Ae. aegypti* produced ~25% more sperm than small males within the same age group. They also reported variation in total sperm capacity as a result of aging. The number of spermatozoa in virgin 1 day old males was about one third of that found in 10 day old males from the same strain. Interestingly, they noted variation in sperm capacity among their laboratory strain and a field strain of the same species, despite both strains being reared on the same adult diet. Fifteen day old males from the field strain produced as many sperm as 25 day old males from the laboratory strain. The authors suggested that this observation must have stemmed from differences in larval rearing conditions, since the males from the field strain were collected while in the pupal stage.

3.1.4 Male nutrition and reproductive success

In order to understand the correlation between male diet and fitness this discussion must turn to the several lines of evidence reported by Yuval *et al.* on Mediterranean fruit flies (Diptera: Tephritidae; Warburg and Yuval 1996, 1997, Blay and Yuval 1997, Yuval *et al.* 1998, Field and Yuval 1999). These studies provide ecologically relevant insight which may further our understanding of how diet can shape the physiology of male insects and potentially their reproductive success. However, it is important to recognize a key difference in the mating system exhibited by Tephritidae, namely lekking behaviour. Briefly, it consists of males defending individual leaves on host trees as mating territories and attracting females to their perch by producing pheromones (Shelly *et al.*, 2002).

Blay and Yuval (1997) examined the effect of supplementing proteins in the diet of male Mediterranean fruit flies. Their prediction was that these dietary differences would manifest in some aspect of the male physiology and/or behaviour directly or indirectly via their respective mates. To quantify any potential differences they measured reproductive success in terms of: 1) number of copulations, 2) amount of sperm transferred, and 3) renewal of female receptivity to second mates. They found that protein-fed males had significantly higher reproductive success across all parameters tested (i.e., more copulations, greater sperm transfer and prolonged refractory period of mates) than sugar-fed controls. Interestingly, the ability of protein-fed males to mate more frequently has since been attributed to a greater pheromone output (Kaspi *et al.* 2002), an essential component of the male fruit fly's courtship. Shelly and Dewire (1994) who studied the correlation between nutrition and copulatory success reported similar findings in oriental fruit flies. In that study, males fed on methyl eugenol (a pheromone component) exhibited longer courtship displays (higher levels of wing-fanning) and attracted more females per minute than did control males.

In the field, lekking males were heavier and contained greater amounts of sugar and protein than resting males (Shelly *et al.*, 2002). There is an energetic cost associated with lekking; nutrient levels declined throughout the day for field-collected, lekking males. Additionally, in no-choice, laboratory trials, females mated more readily with protein-fed than protein-deprived males. The effects of nutritional history even appeared to affect sexual receptivity to further mating. Females that were first mated to protein-deprived males were more likely to remate than females first mated to protein-fed males. The artificial diets used by Shelly *et al.* (2002) simulated a variation in protein content that exists among different natural sugar sources (e.g. nectar and honeydew) and the results demonstrated the influence of diet composition on the reproductive success of male Mediterranean fruit flies (Shelly *et al.*, 2002). In light of the fact that adult male mosquitoes rely solely on sugar meals it is reasonable to predict that diet variation may have similar consequences in terms of their reproductive success.

Although not as comprehensive as their work with Tephritidae, Yuval *et al.* (1993) conducted a few studies with *An. freeborni*, verifying the link between size and mating success of males in this species. This was measured in terms of prolonged swarming duration of large males relative to smaller males. Our lack of knowledge in this field prompts the following questions: Does variation in adult diet influence male reproductive success in mosquitoes? Given the conflicting interests of males and females in reproduction, what components of female life history would be affected by this variation? A brief review of the trade-offs between reproductive effort and survival may provide candidate measures of female fitness susceptible to change under different mating regimes.

3.1.5 Study objectives

Several lines of evidence demonstrate that female insects can suffer significant direct costs of mating (reviewed in Arnqvist and Nilsson, 2000). The cost of reproduction for females is characterised by a reduction of subsequent survival and late reproduction as a result of increased early reproduction (Reznick 1985; Clements, 1999; Dao *et al.*, 2010). In the present study, we test the hypothesis that adult diet affects the reproductive success of male mosquitoes. As in the prior study, males were fed on artificial nectar or artificial honeydew. We then determined whether males induce a greater egg output (lifetime fecundity) in their mates if they were reared on one diet or the other. With the fitness trade-offs to mating in mind, we also compared the survival of females following a single mating with males reared under different diet regimes. To verify that any observed differences in these two parameters were the result of mating itself, we compared the accessory gland profiles of males under each diet regime and spermathecal profiles of their respective mates using one-dimensional gel electrophoresis.

3.1.6 Study model

The genus *Culex* represents a diverse range of mosquito species and from a global public health perspective, one of its most important groupings is the *Cx. pipiens* subgroup that includes *Cx. pipiens* form *pipiens* and *Cx pipiens* form *molestus* (Lee *et al.* 1980; Kassim *et al.* 2012). There is controversy regarding the taxonomic composition of the *Cx. pipiens* group, and some degree of uncertainty in particular surrounding the genetic differences between *Cx. pipiens pipiens* and *Cx. pipiens molestus* (hereafter, *Cx. molestus*) which exhibit both biological and ecological differences (Vinogradova 2000). The two forms do not seem to be genetically isolated and have been reported to hybridize in the United States (Fonseca *et al.*, 2004).

The organism used here, *Cx. molestus*, was first described from Egypt as a unique species by Forskal in 1778 (Knight *et al.* 1951; global distribution shown in Figure 2.1, Smith

and Fonseca, 2004). *Cx. molestus* are distinguished from *Cx. pipiens* based on consistent differences in the expression of facultative autogeny, stenogamy and blood host preference (with *Cx. molestus* biting humans avidly and *Cx. pipiens* preferring avian hosts; Kassim *et al.*, 2012a). Intriguingly, Kassim *et al.* (2012a) found that *Cx. molestus* females can be maintained on sugar alone and that sugar feeding can supplement multiple autogenous egg rafts. However, the number of eggs produced following a blood meal is roughly twice that of autogenous egg rafts, suggesting that autogenous eggs may be the product of suboptimal nutrition status. The authors also reported that *Cx. molestus*, unlike other members in its subgroup, is homodynamic (i.e., it can breed all year round and is cold tolerant). This evidence was based on the observation that the majority of the *Cx. molestus* placed in the outdoor environment with a mean temperature of 12.1°C survived until the adult stage. Additionally, females retained the ability to lay autogenous egg rafts in the subsequent generation of adults suggesting that they may have a greater capacity to oviposit autogenously than is typically expected of facultatively autogenous mosquitoes.

In the prior study, mating was shown to elicit a vitellogenic response in the obligately anautogenous *An. stephensi*. However, this was true only if the females had taken a blood meal. To elucidate whether mating is more than a synergistic stimulus in the process of egg production, it is necessary to consider a facultatively autogenous model, such as *Cx molestus*. While its reproductive strategy is not contingent on blood, it exhibits feeding patterns in the field that can facilitate the transmission of avian and mammalian pathogens (e.g. West Nile Virus) and thus is of great medical interest (Kassim *et al.*, 2012a).

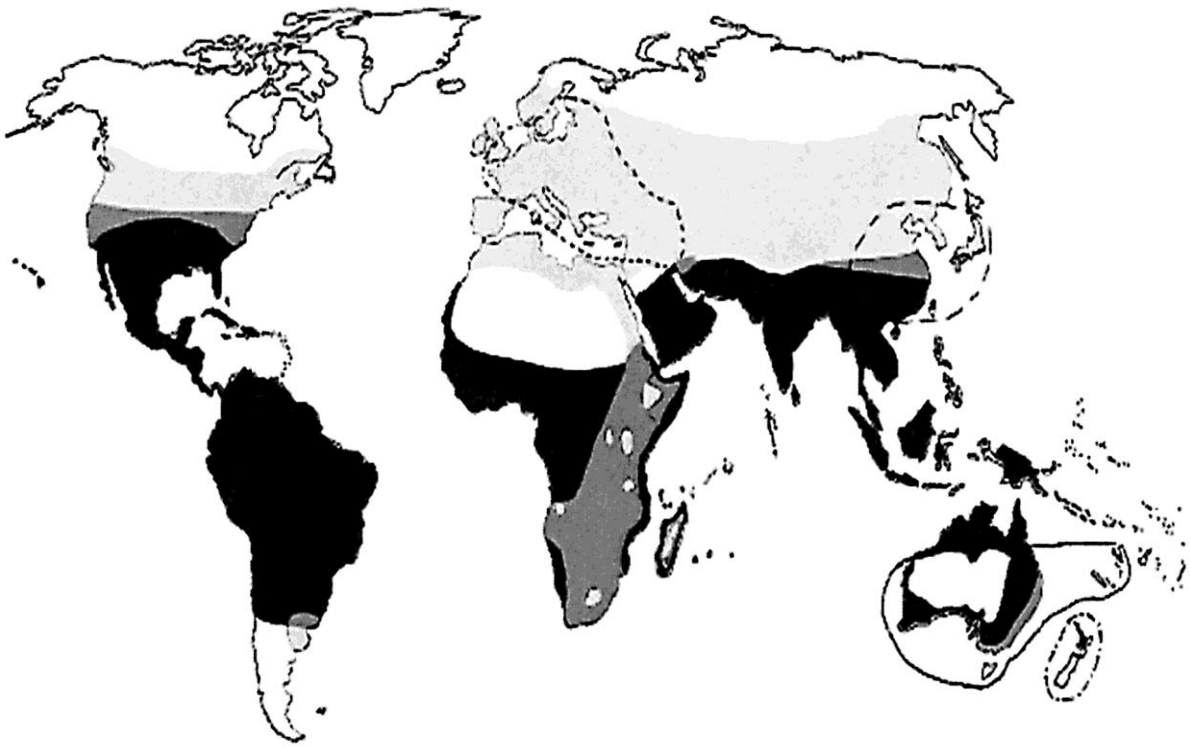

Figure 2.1. Distribution of the *Culex pipiens* complex and its sibling species. Light gray = *Cx. pipiens (molestus)*; black = *Cx. quinquefasciatus*; dark gray = overlapping ranges of *Cx. pipiens* and *Cx. quinquefasciatus* (obtained from Smith and Fonseca, 2004)

3.2 Methods

3.2.1 Mosquito rearing

Cx. molestus stock colony was maintained at 22^{o}C, 30-50% relative humidity and a 12L: 12D photoperiod. Both males and females were housed in collapsible breeding cages (BioQuip, CA 90220) measuring 30 cm^3 and given unrestricted access to a diet of 10% (w/v) sucrose solution. Eggs were laid onto moistened filter paper and larvae were hatched into large plastic trays (46 x 32 x 4cm, BioQuip, CA 90220). Larval were reared on a standard diet (finely ground powder, LagunaTM Goldfish and Koi Chow, Rolf C. Hager (USA) Corp. Lansfield, MA). Larval density was maintained at 120-150 individuals per tray with water depth maintained at approximately 3 cm.

For all experiments, pupae were separated into individual 20 mL scintillation vials with 8-10 mL of water and allowed to emerge into a separate cage. Newly emerged adults were sexed within 8 hours of emergence. Males were isolated from females by placement in same-sex cages containing either 10% (w/v) sucrose solution, artificial nectar (AN) solution or artificial honeydew (AH) solution for the first 5 days following their emergence. Females were also housed in same-sex cages and were given unrestricted access to only 10% (w/v) sucrose solution. From personal observation, >98% of adult *An. stephensi* died after 3 days without sugar.

3.2.2 Diet composition

The artificial sugar diets used in all the experiments were intended to simulate naturally occurring nectar and homopteran honeydew (as in the prior study, with *An. stephensi*). The artificial nectar (AN) diet was composed of 4.0% (w/v) fructose, 4.0% (w/v) glucose, and 2.0% (w/v) sucrose. The artificial honeydew (AH) diet was composed of 6.0% (w/v) fructose, 4.0% (w/v) glucose, 2.0% (w/v) sucrose, 8.0% (w/v) melezitose, 0.1% (w/v) D-asparagine, and 0.1%

(w/v) D-glutamine. All diets were prepared separately in sterile conical flasks, stored in a refrigerator and presented to the test animals on a loosened dental wick protruding from the top of 10 mL glass shell vial.

3.2.3 Oviposition assay

Pupae were collected from the stock colony and allowed to emerge in a new breeding cage. Adults were sexed within the first 8-10 hours of emergence and males were transferred into same-sex containers for 5 days post-emergence. One of two sugar meals (AN or AH) was placed in each container and replenished daily until males were introduced to their mates. All test females were also housed in same-sex cages for 5 days post-emergence with unrestricted access to 10% (w/v) sucrose solution and water.

At 5 days post-emergence females were presented with an excess of mates (2:1) for 8 to 10 hours to allow mating. A total of 30 females were presented to each group of males (mates of AN-fed males, n= 30; mates of AH-fed males, n= 30). After 8 to 10 hours, males were aspirated out of the breeding cages and the total number of eggs laid by females in each mating regime was recorded every 48 hours until egg production had ceased. All sugar solutions were replenished daily until the end of the experiment. Lifetime fecundity was chosen as a measure of reproductive success because mating experiments conducted within the confines of breeding cages are subject to high mate encounter rates, making other measures of reproductive success (e.g. insemination rate of males) unreliable. Insemination rates of laboratory based colonies have been shown to increase when enclosure volumes were reduced (Stone *et al.* 2009). In the present study, the male to female sex ratio was intentionally skewed to ensure mating had occurred.

3.2.4 Confirmation of insemination status

To confirm whether or not females had mated, their spermathecae were inspected for the presence of sperm. Females were first aspirated and freeze-killed by placement in -20^{o}C freezer for 10 minutes. Under a microscope (Leica MS5, Leica Microsystems) each female was then dissected on a glass slide in a drop of 1X phosphate buffered saline (PBS). The spermathecae (3 in *Cx. molestus*) were removed from the last abdominal segment and inspected for presence of sperm under a compound microscope (Alphaphot-2 YS2, Nikon) following the technique described by Benedict (2007). Only inseminated females and individuals that survived until the end of the experiment (mates of AN-fed males, n=26; mates of AH-fed males, n= 22) were used in data analysis.

3.2.5 Survivorship assay

To compare the survivorship of females under different mating regimes all females were aged to 5 days post-emergence and then presented to an excess of mates (>2:1) for an 8 to 10 hour period. Males were then removed from the breeding cages. The mates of AN-fed males (n=20) were left in the same breeding cage and allowed to lay eggs on damp filter paper with unrestricted access to 10% sucrose (w/v) and water. This same procedure was carried out with the mates of AH-fed males (n=20).

Virgin females were isolated to same-sex cages within 8 to 10 hours of emergence and given unrestricted access to 10% sucrose (w/v) and water. All females (n= 20 per treatment) were monitored daily, with food and water being replaced as necessary until all individuals had died. This procedure was repeated 3 times, yielding a total of four replicates. Insemination status of mated females was confirmed post mortem. Individuals that were not inseminated were excluded from the analysis.

3.2.6 Accessory gland protein profile comparison

Adult males were transferred into same-sex cages for the first 5 days post-emergence with *ad libutum* access to either AN or AH. Sugar meals were replenished daily until dissection. To verify that male AGPs are transferred to females during mating, the spermathecae of recently mated females and virgin females were included in the analysis. All females were aged for 5 days post-emergence in same-sex cages and given access to only 10% sucrose. It was important to ensure females were of the same age, since age has been shown to influence receptivity to copulation and responsiveness to insemination in *Cx. molestus* (Lea and Evans, 1972). Mated females were obtained by placing virgin females in cages (n=20 females per cage) with an excess of males (n= 40 males per diet treatment) for an 8 hour period. After this period, the test animals were prepared for dissection.

3.2.7 Protein extraction and separation assay

All males and females were aspirated out of their cage and killed by placement in a -20^{o}C freezer for 10 minutes. Mosquitoes were individually placed on a glass slide in a drop of 1X PBS under a microscope (Leica MS5, Leica Microsystems). The accessory glands were removed from each male by carefully pulling the last abdominal segment away from the remainder of the body. The glands of virgin males were visible as faint yellow sacs attached to the ejaculatory ducts near the terminalia, while those of mated individuals appeared as white sacs. Lower reproductive tracts from virgin or mated females were dissected out by carefully pulling the last abdominal segment away from the remainder of the body and then removing tissues surrounding the bursa and the three spermathecae. Once excised the accessory glands of 15 males per diet treatment were placed in an Eppendorf tube containing 40 µL of chilled 1X PBS (80mM NaCl, 10mM KCl, 1mM Na_2HPO_4, 0.2mM KH_2PO_4; pH 7.2). Following dissection, the same procedure was repeated with the spermathecae of 15 mated females per

mating regime (i.e., n = 15 for both mates of AN-fed males and mates of AH-fed males) and 15 virgin females. Samples were then centrifuged at 15,000 g for 15 minutes to separate soluble molecules from cells and tissue fragments. Supernatants (soluble fraction) were removed and placed in new tubes. Pellets were resuspended in 10 µL of chilled sample buffer and centrifuged again for 15 minutes at 15,000 g. The supernatant was added to the previously isolated soluble fraction and stored at 4^{o}C for 6 hours. Then 10 µL of each sample was added to a tube with 10 µL of 2X Laemmli SDS sample buffer (0.5M Tris–HCl pH 6.8, 20% glycerol, 10% SDS, 10% β-mercaptoethanol, 0.5% Bromophenol blue). Samples were heated to 95^{o}C for 5 minutes and soluble proteins in the supernatant were separated on a one-dimensional 10% gradient SDS-polyacrylamide precast gel (Mini-PROTEAN®TGX^{TM} Gels, BioRad) at 150 V for 40 m. Samples were then stained with Silver Stain (BioRad) for visualization of the bands according to the manufacturer's instructions.

3.2.8 Statistical analysis

Data from the oviposition assay and survivorship assay were tested and conformed to the assumptions of parametric tests (i.e., normality and homogeneity of variances). Wilks-Shapiro test was used to test for normality. Homogeneity of variances was tested using Levene's test. Independent samples t-test was then used to determine any significant differences in average lifetime fecundity ($\boldsymbol{m}_x$) among different mating regimes. Kaplan Meier curves and a log rank test were used for the analysis of survivorship ($\boldsymbol{l}_x$) amongst the females in each mating regime. In all instances, values of $p < 0.05$ were considered statistically significant.

All statistical analyses were conducted using IBM SPSS Statistics Version 20 (IBM Corporation, New York). Data are presented as mean ± standard error (SE) unless indicated otherwise. Figures were plotted using Microsoft Excel (2010; Microsoft Corporation, Washington)

3.3 Results

3.3.1 Confirming insemination status

Once egg production had ceased, females were dissected and their spermathecae were scored for the presence of motile sperm. Of the 30 females exposed to AN-fed males, 87% (n=26) were successfully inseminated. With the same male to female sex ratio (2:1), sperm was detected in 73% (n=22) of the females exposed to AH-fed males. Only gravid females were used in the statistical analysis.

3.3.2 Accessory gland protein profile comparison

Proteins from male accessory glands and ejaculatory ducts and virgin and mated female bursae inseminalis and spermathecae were acquired by centrifuging tissue homogenates of 15 individuals of each class for 15 min and then subjecting the entire supernatant to electrophoresis on a 10% SDS-polyacrylamide precast gel. Boxes in Figure 2.2 indicate specific bands that were exogenous to females.

Note that some bands were either absent (e.g. 28-33 kDa range and 110-116 kDa range) or lighter (e.g. 36-38 kDa range) in the mated female and virgin female samples than that seen in accessory gland extracts of the corresponding male group.

3.3.3 Male diet composition and female lifetime fecundity

Average lifetime fecundity was used to compare reproductive success (i.e., the ability to induce oviposition) of females under each mating regime. The effect of male diet on female fecundity was therefore evaluated by comparing the mean number of eggs laid by females in each mating regime (AN_m, n=26; AH_m, n=22) until the onset of reproductive diapause. Female lifespan was monitored daily until all individuals had died to ensure that no further egg production occurred.

An independent samples t-test was also used to compare the lifetime fecundity of the females in each mating arrangement. There was a difference in mean number of eggs produced over the lifetime of the females under each mating arrangement (Independent samples t-test; $t(46) = 5.05$, $p < 0.001$). The mates of nectar-fed males produced ~11% more eggs than their counterparts (Figure 2.3). Additionally, these females did not enter reproductive diapause until after 19 days post-emergence, while egg production was halted in the mates of honeydew-fed males after 17 days post-emergence (data not shown).

Initially, daily fecundity was considered for use as an indirect measure of male reproductive success; however, the limited sample size of this particular experiment would have produced misleading estimates of late reproduction (i.e. days 15 to 19). This is because estimates of late reproduction would have been derived from a progressively smaller pool of individuals. By using lifetime fecundity artifacts of limited sample size could be reasonably minimized. However, these findings could benefit from additional replicates to ensure that the observed trend in female fecundity is reflective of a true advantage in reproductive competence imparted by the nectar meal and not an artifact of limited sample size.

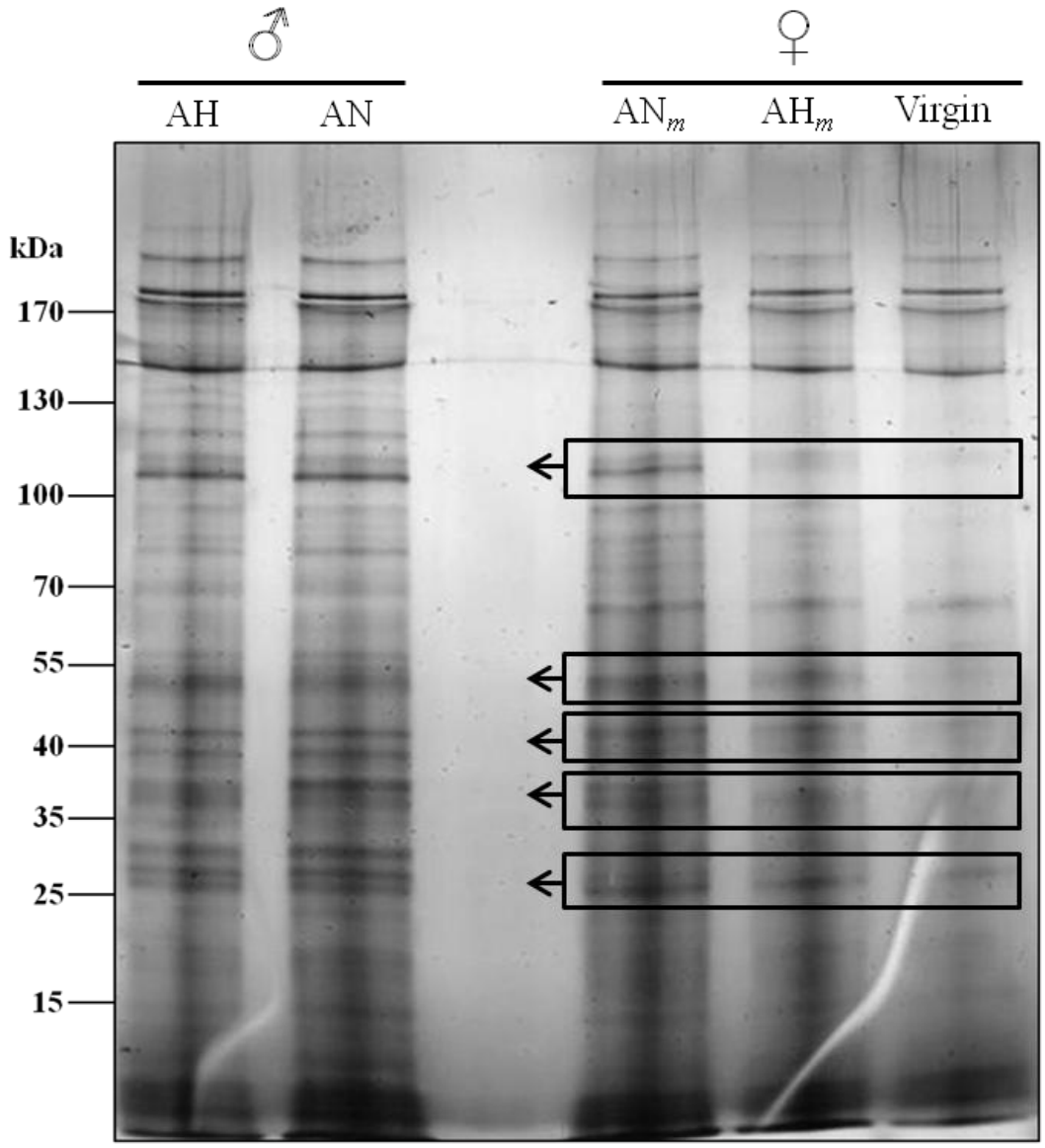

Figure 2.2. Proteins from male *Culex molestus* accessory glands and ejaculatory ducts and virgin and mated female spermathecae and bursae inseminalis. Boxes indicate protein bands that are present in the males (AH, artificial honeydew-fed males; AN, artificial nectar-fed males) but absent or lighter in the mated female samples (AN$_m$, mates of nectar-fed males; AH$_m$, mates of honeydew-fed males) and the virgin female (control) sample. Samples were acquired by centrifuging tissue homogenates of 15 individuals of each class for 30 min and then subjecting the entire supernatant to electrophoresis on a 10% SDS-polyacrylamide gel.

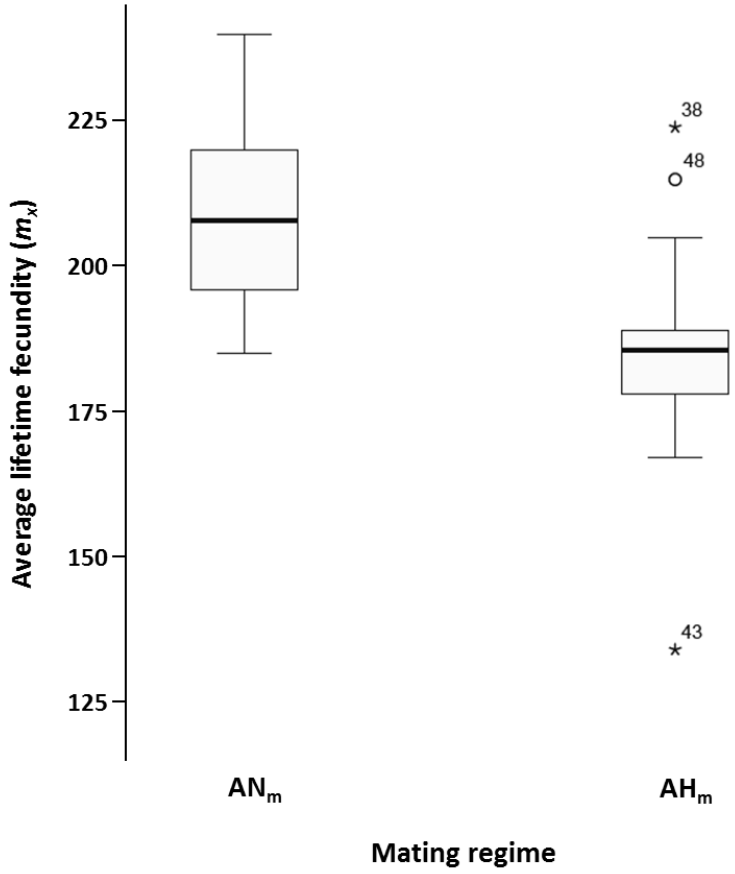

Figure 2.3 Effect of mating on lifetime fecundity of *Culex molestus*. Fecundity was measured as the average number eggs laid per female **(m_x)** under a given mating arrangement (mates of nectar-fed males, n=26; mates of honeydew-fed males, n=22) until the onset of reproductive diapause. Independent samples t-test indicated that there was a significant difference (t (46) = 5.05, $p < 0.001$) in the average lifetime fecundity of the two groups of females; with mates of nectar-fed males (AN_m) producing 208.9 ± 15.8 eggs (SD), while those of honeydew-fed males (AH_m) produced 184.3 ± 18.1 eggs (SD). Circles (^{o}) indicate outliers and asterisks (*) indicate far outliers, both of which were included in the statistical analysis.

3.3.4 Male diet composition and female survival

One of the costs of reproduction for female mosquitoes, and many insects, is a reduction of subsequent survival. This has traditionally been thought to be a consequence of an elevated oviposition rate (Barnes and Partridge, 2002). To test whether male nutritional history affects female post-mating survivorship, the longevity of females mated with nectar-fed males and honeydew-fed males were compared (Figure 2.4). There was no difference (Log Rank test: X^2 = 0.238, d.f.= 2, p= 0.888; n= 20 females per mating regime) in the survival functions of females that were mated with nectar-fed males (M=18.65 ±1.23 days), honeydew-fed males (M=19.40 ±1.20 days) and controls (virgins, M=18.30 ±1.18). Three additional replicates were set up in this experiment for each mating regime (n= 20 females per mating regime; n=60 per replicate). No differences in survival time were observed across the remaining three replicates (Replicate 2: Log Rank test: X^2 = 3.680, d.f.= 2, p= 0.159; Replicate 3: Log Rank test: X^2 = 7.946, d.f.= 2, p= 0.190; Replicate 4: Log Rank test: X^2 = 2.508, d.f.= 2, p= 0.285).

Of the mated females that were scored for the presence of sperm in this experiment (in total n= 80 females per mating regime) there were 76 and 77 females successfully inseminated females by nectar-fed males and honeydew-fed males, respectively. The high insemination rate (95% for AN_m and 96% for AH_m) observed in this experiment can be attributed to the high male to female ratio (>2:1) used. Interestingly, virgin females exhibited similar survival as mated females. Comparing the survival of virgin females with that of uninseminated females exposed to mates would have allowed determination of the costs incurred by female *Cx molestus* that were non-receptive to mating. However, the high rate of insemination in this particular experiment (95-96%) resulted in too few uninseminated females (< 4 per mating regime) to provide meaningful statistical analysis.

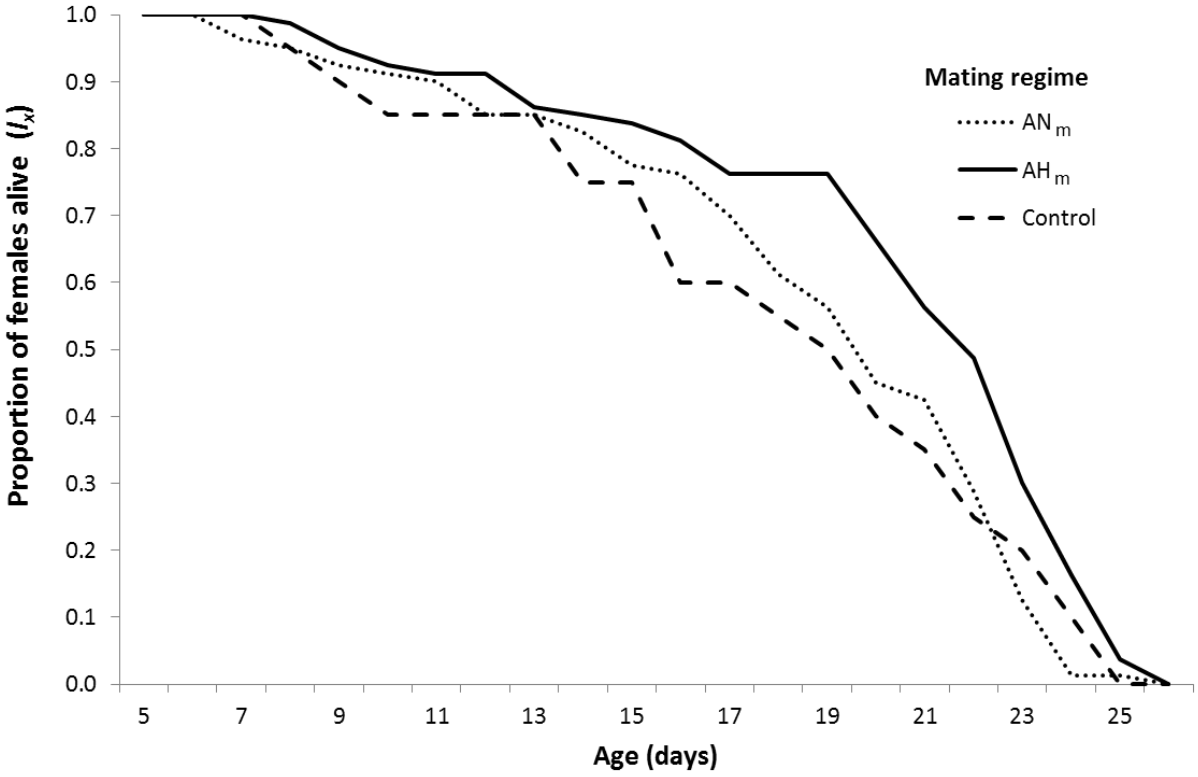

Figure 2.4. Effect of mating on survivorship of female *Culex molestus*. Survivorship ($\boldsymbol{l_x}$) was computed as the proportion of females alive on each observation date until all individuals had died. Kaplan Meier curves in combination with log rank test were used for the analysis of survival amongst the three groups of females. There was no difference (Log Rank test: X^2 = 0.238, d.f.= 2, p= 0.888) in the mean lifespan of females mated with nectar-fed males (AN_m, n=20), honeydew-fed (AH_m, n=20) and controls (virgins, n=20).

3.4 Discussion

The reproductive success of the male mosquito is largely determined by successful insemination. For a given male in a population, this parameter can be measured in two ways: (1) as the proportion of females that contain his spermatozoa in their spermathecae or (2) as the number of eggs produced by his mates. The latter measure applies especially when considering those species in which females generally mate once, making the first-mating male the sire of all the offspring. To date, several factors have been shown to increase male copulation and insemination success, including adult size at emergence (Verhoek and Takken, 1994), age (Mahmood and Reisen, 1994) swarm density (Voordouw *et al.*, 2008), the male to female sex ratio (Charlwood and Jones, 1979), and AGPs in the seminal fluid (Dickinson and Klowden, 1997; Klowden 2001, reviewed in Baldini *et al.*, 2012). As mentioned earlier, one study has even demonstrated the link between sugar feeding (specifically from nectar sources) and male reproductive success (Yuval *et al.*, 1994). In that study, however, nectar sugars were only implicated in their capacity to fuel flight and support swarm attendance. Perhaps the best demonstration of how sugar availability influences the insemination success of wild mosquito populations comes from the work of Stone *et al.* (2009). The authors compared the insemination rate of male *An. gambiae* under various diet regimes in large walk-in mesocosms that simulated field conditions. They found that sucrose-fed males were able to successfully inseminate nearly 5 times as many females than sugar-deprived males. Although that study highlighted the importance of sugar meals on male reproductive success, wild populations can rarely be established in areas with virtually no sugar source.

3.4.1 Male diet and female lifetime fecundity

The present study examined the reproductive success of males under different diet regimes. These artificial diets were designed to simulate naturally occurring nectar and honeydew. The reproductive success of males under each diet regime was then measured in terms of the lifetime fecundity (i.e. lifetime egg output) of their mates. It was found that females mated with nectar-fed males had a significantly greater (~11%) lifetime fecundity that those mated with honeydew-fed males (Figure 2.3). Although, all females were presented with the same diet (10% sucrose) variation in size, age and larval reserves were not effectively controlled in this experiment. It is reasonable to expect some variation even when individuals are harvested from the same generation and within a short time interval. Further data must be obtained in order to ascertain whether the differences in female lifetime fecundity can be safely attributed to the differences in the males' nutritional histories.

There is evidence that suggests increased egg production may be influenced by the quality of seminal fluids (include AGPs, steroidal hormones and sperm) transferred during mating. This notion gains support from the work of Isaac *et al.* (1999) who investigated the effects of a specific male accessory gland protease called angiotensin-converting enzyme (ACE) in *An. stephensi*. The authors administered ACE-inhibitors in the glucose diet of males and then allowed them to mate with blood-fed females. They found that the mates of glucose-fed males (controls) produced 80% more eggs than those mated with ACE-deficient males. Although, their findings do complement the findings of the present study, direct comparisons of the observed post-mating responses would be misleading due to differences in reproductive strategy of the model species used. Females of *An. stephensi*, exhibit obligate anautogeny (i.e. require blood to produce eggs), while those of *Cx. molestus* exhibit varying degrees of facultative autogeny (Becker *et al.* 2003). The influence of male-derived substances on female

fecundity would be greatly overestimated in the former since egg production is triggered mainly by the ingestion of blood.

Using basic one-dimensional gel electrophoresis, the present study also compared the accessory gland profiles of males under varying diet regimes and the spermathecael profiles of their respective mates shortly after mating (≤ 8 hours, Figure 2.2). While the quantity of AGPs cannot be assessed based on these findings, the absence of specific male-derived proteins in mates of honeydew-fed males (e.g. 110 kDa highlighted by uppermost box in Figure 2.2) does provide potential candidates to test, using more qualitative proteomics, for their role in modulating female fecundity. Interestingly, the accessory gland profiles of honeydew- and nectar-fed males appeared to be similar. However, comparison of the spermathecal profiles of their respective mates suggests that the ability of males to transfer AGPs may vary depending on their adult diet. This is akin to the finding that male sperm capacity in some species varies depending on resource availability during the larval stage (Ponlawat and Harrington, 2007).

It is thought that the AGPs of insects evolve more rapidly than both female-specific and non-reproductive proteins in general (Panhuis and Swanson, 2006; Haerty et al, 2007). This accelerated level of divergence is observed even within members of the same genus. Haerty *et al.* (2007), for example, found that there were fewer homologs of *Drosophila melanogaster* AGPs across 12 *Drosophila* species than any other non-AGPs. Their evolution, like that of most sex-specific traits, may be driven by mechanisms such as cryptic female choice (Wolfner, 2009). Cryptic female choice and other evolutionary mechanisms (e.g. sperm competition) can potentially drive speciation; however, it is difficult to determine the extent of their influence in mosquitoes given the limited data available on female remating in the field. To date, female mating patterns have been investigated in only a few anopheline populations. Recent reports suggest extremely low rates of remating (ranging from 1-4%) in females of *An. gambiae* (Tripet *et al.*, 2003) and *An. freeborni* (Yuval and Fritz, 1994). Females of *An. nuneztovari*

exhibit relatively high rates (15%, Yuval 2006), whereas those of *An. dirus*, *An. maculatus*, and *An. messae* were found to not exhibit polyandry at all (Yuval 2006).

Quantitative differences in the amount of seminal fluids transferred during mating can also explain the differences observed in female fecundity. Klowden (2006) conducted an experiment in which the spermathecae of mated *An. gambiae* and *Ae. aegypti* females were manipulated to determine whether post-mating responses (oviposition behaviour, in this case) was mediated by nervous signaling. Interestingly, it was found that while oviposition in *Ae. aegypti* was triggered by specific peptides transferred from male accessory gland secretions, the mechanism in mated *An. gambiae* instead appeared to be triggered by the presence of sperm in an innervated spermatheca. Klowden found that when previously mated *An. gambiae* had their spermathecae surgically removed they were unable to oviposit, but *Ae. aegypti* females treated similarly were able to lay infertile eggs. This suggests that nervous signals received from spermathecae replete with seminal fluid may, at least in some species, trigger oviposition.

One relevant question that was not addressed in the present study is whether the spermathecae of mated females would reflect varying protein profiles as they age. Logic dictates that quantity of seminal fluid would decrease with each oviposition cycle, but the fact that seminal fluid proteins undergo proteolytic cleavage within the female reproductive tract (reviewed in Wolfner, 2009) suggests that the differences may be more qualitative in nature. In *D. melanogaster*, for example, the half-life of seminal fluid proteins can range from a few hours (Ravi *et al.* 2005) to 4 days post-mating (Peng *et al.* 2005). One protein, called sex peptide, induces oviposition in *D. melanogaster*. It is found in the spermathecae bound to sperm where it is gradually released over time (Peng *et al.* 2005). By prolonging post-mating responses like oviposition, seminal fluid proteins may allow male *D. melanogaster* to maximize their reproductive potential.

All this evidence brings to mind the following question: What mechanism underlies the post-mating response observed in mosquitoes? A full answer to this question requires a deeper understanding of the factors that cause variation in reproductive success of males. Aside from energy reserves acquired during the larval stage, for male mosquitoes, dietary sugar composition is the only other variable subject to manipulation, be it by the experimenter in the laboratory or by resource availability in the field. Data on the ecological significance of male diet preference, however, is scant at best. Recent experiments with *Cx. quinquefasciatus* (Tomberlin *et al.*, 2006) and *Cx. molestus* (Jhumur *et al.*, 2006) demonstrated that young adults of both sexes could learn to associate odours (e.g. vanilla extract and plant kairomones such as phenylacetaldehyde) with the presence of sugars. This learning ability appears to be age-dependent, as trained individuals of both species exhibited greater responsiveness to volatiles than their untrained counterparts (Tomberlin *et al.*, 2006; Jhumur *et al.*, 2006). The ability to distinguish between different plant nectars would certainly be advantageous for males in wild populations as it would allow adjustment to different plant communities.

Studies of mosquito population dynamics apply optimal foraging theory to predict whether or not an animal (most frequently the female of the species) should include a particular item in its diet based on its caloric value, resource availability, and handling-time cost (Stephens and Krebs 1986; Roitberg and Mangel, 2010 and references therein). Models based on this theory attempt to predict mosquito biting rates and host-seeking behaviour mainly to further disease intervention efforts (e.g. malaria in Roitberg and Mangel, 2010). The utility of different food sources are not typically weighed in terms of their ability to maximize lifetime reproductive output and/or survival. Notably, some models based on this theory do emphasize the importance of nectar-bearing plants, but only in their ability to support female host seeking behaviour (Roitberg and Mangel, 2010). But the fact that sugar feeding plays a significant role

in male reproductive capacity (e.g., sperm production) is overlooked; even more so is the fact that males exert considerable influence on female physiology and behaviour through mating.

One study, for example, demonstrated that differences in protein content of accessory glands brought about by suboptimal male nutrition affected the host-seeking behaviour of female *Ae. aegypti* (Fernandez and Klowden, 1995). A greater proportion of the females mated to starved males responded to host cues than those mated to sugar-fed males. This suggests that there are more variables contributing to female host-seeking behaviours and biting rates than those accounted for in models based on classical optimal foraging theory.

3.4.2 Male diet and female longevity

In comparing the survivorship functions of females under different mating regimes (Figure 2.4), it was observed that not only did mates of AN-fed males and AH-fed males share similar survival patterns, but so did virgin females. Admittedly, these results are inconsistent with the widely recognized trade-offs between somatic maintenance and reproductive output (i.e., cost of egg production and oviposition; reviewed in Stearns 1992). But partitioning the cost of reproduction into its constituent components may offer a relevant explanation for this observation.

For female mosquitoes that adopt autogeny, the costs of reproduction include the effects of the following: 1) male courtship behaviour, 2) the act of mating, 3) sperm and male seminal fluids, 4) egg production and 5) oviposition. An additional cost exists for anautogenous species that is associated with egg development after blood feeding (Stearns 1992; Dae *et al.*, 2010). For the virgin controls in this experiment, these costs of reproduction were virtually non-existent since they were isolated within 8 hours of emergence. Early isolation is important in these types of mating experiments because after the first 12 hours of adult life, male sexual maturation begins and their sexual organs and antennal fibrillae can facilitate mating (Howell

and Knols, 2009). Although, the females in the experimental treatments were mated, it is likely that the short duration of exposure to males (8-10 hour period) minimized the costs associated with mating (e.g. those associated with courtship behaviour and copulation). Lending support to this argument is the work of Dae *et al.* (2010), who evaluated the costs of reproduction in *An. gambaie* by comparing the survival of mated and uninseminated females that were allowed access to males for 2 days and virgin controls (no access to mates). The experimenters found virgin and mated females survived longer than uninseminated females with access to mates but their survival did not differ significantly from one another. They concluded that the energetic costs of being non-receptive to courtship must represent the most costly component of female reproduction. Again, it is important to keep in mind that these insemination rates are subject to artifacts and may have little relevance to natural conditions. For example, the *An. gambaie* males used in Dae *et al.* (2010) were only able to inseminate 52% of the females after 2 days of exposure. Male *Cx. molestus,* on the other hand, were far more likely to mate in confined spaces as they were able to inseminate 73 to 87% of the females with only 8 to 10 hours of exposure (personal observation).

Evaluating the effect of male nutritional history on the longevity of their mates, may not have been appropriate in this case since laboratory environments are less energetically demanding that natural ones. Future studies should incorporate other measures of reproductive success to compare the role of varying diet regimes. Candidate measures include egg hatch rate and larval survival rate as they are not prone to overestimation in laboratory studies. Alternatively, large-scale studies which utilize indoor mesocosms can be used to more closely mimic field conditions. Stone *et al.* (2009) constructed a mesocosm that provided sufficient room for swarming, sugar-bearing plants, a human host and even predators. In these semi-field conditions, the effects of varying diet regimes on spatially limited components of male fitness

(e.g. mating frequency, swarming activity, and female receptivity) can yield ecologically relevant data.

3.5 **Summary**

Male and female mosquitoes exhibit diverging interests with regard to reproductive investment. The present study examined the indirect effects of diet composition on two female life history traits: lifetime fecundity and survivorship. Two variables that may have potentially confounded fecundity estimates in this study are individual differences in larval reserves at emergence and cross-generational differences in female oviposition behaviour. The females used to in this study were obtained from the same generation and reared under identical conditions as larvae thus it is reasonable to expect that they shared a similar capacity to produce autogenous eggs. While the notion that nectar feeding may impart some reproductive advantage to male mosquitoes is complemented by the observations of this study (i.e., difference in life-time fecundity), these results should be treated with caution, and mostly indicate male diet as a candidate regulator of mosquito fecundity under optimal conditions. These findings should encourage further exploration of such effects in natural settings.

If sugar quality in nature can regulate male reproductive fitness through its subtle influence on female egg output, it may ultimately impact the size of a given population. The management of local sugar sources could potentially be used as a means of population control. Wäckers *et al.* (2007) proposed that parasitoid food sources could be introduced into agricultural lands to support biological control of pests. Similarly, introducing artificial hosts that simulate poor sugar sources (Stone *et al.*, 2012) (e.g. sugars that contain AGP-targeting inhibitors) may reduce male fertility and help suppress population growth. However, our current understanding of the factors that influence male nutritional and reproductive ecology in nature is very limited. Before sugar feeding can be exploited to further population control efforts, every female life history parameter regulated by male diet must be identified.

CHAPTER 4

General Discussion

In mosquitoes, and other vectors, population- and disease transmission- dynamics are predicated on the trade-off between survivorship and reproductive output. Theortical concepts such as vectorial capacity were introduced to identify the life history traits most useful in evaluating the propensity of a particular insect population to spread a disease (proposed by Garrett-Jones, 1964, reviewed in Dye, 1992). Vectorial capacity is defined as "daily rate at which future inoculations arise from a currently infective case" (Dye, 1992). The components of vectorial capacity (*C*): vector density relative to humans (*m*), biting frequency (*a*), survival rate (*p*), and duration of the extrinsic incubation cycle of the parasite in question (*n*) are expressed mathematically as (Dye, 1992):

$$C = \frac{ma^2 p^n}{-\ln(p)}$$

Notice that vectorial capacity is directly proportional to survival rate (*p*) and biting frequency (*a*). More over, the fact that these components are magnified by powers of n and 2, respectively, implies that even a small change in either can result in an exponential change in vectorial capacity. Based on the literature reviewed it is easy to see how the different components of vectorial capacity can be affected by sugar feeding. Sugar meals have been shown to improve insemination performance, which can indirectly enhance the fitness-related component, density (*m*), by accelerating egg production in environments where female food resources (sugar and/or blood) are not limiting. The biting rate (*a*) is affected by sugar feeding directly, through its inhibitory effect on supplementary blood feeding within a gonotrophic cycle (Foster and Eischen, 1987) and by delaying oviposition (Foster and Eischen, 1987;

Foster, 1995). Lastly, adult survival (*p*) is affected by both the frequency and quality of sugar feeding as is evidenced by their short lifespan in the absence of sugar meals.

That sugar meals are important contributors to vectorial capacity was recently demonstrated in a mark-release-recapture study of *An. sergentii* by Gu *et al.* (2011). The authors found that the presence of the local primary nectar source (from flowering *Acacia* trees) accounted for over a 250-fold difference in *An. sergentii's* vectorial capacity. When the flowering trees were available, *An. sergentii* exhibited greater population size, higher survival rates and shorter duration of the gonotrophic cycle (presumably, due to more frequent blood feeding and oviposition; Gu *et al.*, 2011). The most significant effect of the sugar deficiency on vectorial capacity was a 23% reduction in survival rate in the sugar-deficient compared to sugar-rich sites. This was attributed to the fact that female mosquitoes must be old enough to allow the malaria parasite sufficient time to develop to be able to transmit malaria. What is interesting to note is that, the sugar content of the flowering *Acacia* trees used in that study is far more dilute than typical nectar and honeydew sources (reviewed in Gu *et al.*, 2011). Given that male mosquitoes are obligate sugar feeders, it is of equal interest to know how the differences in the composition of plant and homopteran sugar meals they utilize affects their mating success, post-mating responses of their mates and ultimately, the vectorial capacity of local populations.

The present study is the first to investigate the fitness consequences of AN- and AH- feeding by male mosquitoes, at the molecular and organismal levels. However, the implications of these findings must be taken with caution given the nutrient-rich rearing conditions of our insectaries. Despite the limitations, the approach used here serves as a valid starting point for the study of the reproductive ecology of male mosquitoes. Further experiments that take into account intrasexual variation in body size at the time of emergence (indicator of larval reserves) as well as cross-generational differences in female oviposition

behaviour must be conducted in order to determine the ecological relevance of these trends in the field.

In *Cx. pipiens, Ae. togoi* and *Wyeomyia smithii* nutrient poor larval diets have been reported to limit the expression of autogeny and consequently reduce the size of autogenous egg rafts (Clements 1999; Kassim *et al.,* 2012b). The nutritional regulation of this trait is also evident in *Cx. molestus,* as larvae reared in nutrient rich environments emerge as larger adults (as indicated by significantly greater wing lengths) which produce significantly larger autogenous egg rafts (Kassim *et al.,* 2012b). More importantly, sugar feeding by adults further enhances this propensity to produce autogenous eggs in *Cx. molestus*. Kassim *et al.* (2012b) found that laboratory colonies of *Cx. molestus* with access to sugar produce females which lay significantly more autogenous eggs per raft. In all experiments of the present study, female diets composition were kept constant (10% sucrose solution) so that any differences observed in female fecundity could be attributed to the diet regime of their mates. However, because there is a strong correlation between larval nutrition and body size (Kassim *et al.*, 2012b); the observed trends in lifetime fecundity may be confounded by size variation.

There is also evidence that the autogenous oviposition capacity of female mosquitoes can change substantially over many generations in long standing laboratory colonies. Selective pressure which promotes autogeny is typically applied by restricting blood feeding and supplementing larval diets with amino acids (Lea, 1964). In the facultatively autogenous, *Cx. tarsalis*, selection for autogeny in the laboratory resulted in >3 fold increase in the mean number of eggs per female in just 20 generations (Su and Mulla, 1997).This shift in oviposition capacity was accompanied by a number of other changes including a reduced egg raft size and lower hatch rates. Since the present study sought to compare the effects of mating on specific female lifehistory traits in the absence of blood, the fact that the *Cx. molestus* colony was not blood fed here did not interfere with our objectives. However, the fact that cross-generational

differences in autogenous oviposition capacity exist, meant that differences in reproduction-related traits (e.g. lifetime fecundity and hatch rate) among females from the parental and subsequent generations may not be ecologically relevant. As a result, all mating experiments were conducted using individuals from the same cohort.

Still, the plasticity of *Cx. molestus* and other facultatively autogenous species makes them ideal models for examining exogenous regulators of female reproduction. Understanding the extent to which exogenous factors (e.g. adult sugar meal composition and male-derived substances) can influence the expression of autogeny in such models may allow researchers to accurately assess their role in population dynamics.

Conclusion

This thesis focused on the importance of sugar feeding by male mosquitoes as it relates to the reproductive success of two medically relevant mosquito species, namely *An. stephensi* and *Cx. molestus*. A critical component was to study the effects of varying diet composition on certain female life history traits associated with a key stage of egg production called vitellogenesis. Male were reared on mimics of naturally occurring plant nectar and homopteran honeydew for several days post emergence so that the fitness benefits conferred by each diet (if any) could be assayed without the confounding effects of aging. The upregulation of vitellogenin in females mated with males under each diet regime was compared using the malaria vector, *An. stephensi*. Data suggested that artificial nectar-fed males are able to elicit a significantly greater response (2X) than their artificial honeydew-fed counterparts. However, this effect was only observed in females that had recently blood-fed (Chapter 2), suggesting that mating may be a synergistic stimulus for vitellogenin expression in anautogenous mosquitoes.

This prompted the use of the facultatively autogenous model, *Cx. molestus*, which allowed for an accurate estimation of the effect of mating in the absence of blood. Due to the lack of sequence data for this species, the vitellogenic response of females in response to mating was not investigated. However, other ecologically relevant parameters, such as female lifetime egg production and survivorship were compared following mating. Results of this study were complementary to those of Chapter 3, as artificial nectar-fed males were able to induce greater (11%) egg output in their mates than artificial honeydew-fed males. However, further data are required to validate these findings since they were derived from only a small population of mated females.

Intriguingly, virgin and mated female *Cx. molestus* exhibited similar survivorship functions. Studies have shown that cost of being non-receptive to mating may be high for

female mosquitoes (e.g. shorter lifespan, Dao *et al.*, 2010). The fact that the mated females in this study were only exposed to males for a short period (less than 8 hours) suggests that the deleterious effects typically associated with mating may have been minimized.

Previous studies with *Drosophila* have also shown that there is a strong correlation between accessory gland substances and female reproduction (Wolfner, 2009 and references therein). Interestingly, the accessory gland contents of *Cx. molestus* males in this experiment did reveal varying protein profiles under different diet regimes. Although these findings could benefit greatly from more sensitive proteomics, they do provide some insight on how the suite of AGPs that males produce can vary depending on the composition of their diet. If diet composition in the field similarly affects the suite of AGPs males can produce, individuals that possess olfactory receptors highly tuned to nectar sources would enjoy greater reproductive success. This ability may be further augmented with age, as the male encounters a broader range of flowering plants during its adult life.

Future research should aim to uncover the nature of sugar feeding behaviour of wild populations. Do male mosquitoes actually exhibit diet preference or is sugar feeding driven by the availability and accessibility of sugar meals? Greater insight into what drives this behaviour can be gained by factoring in volatile components of different sugar sources into the equation. By doing so, we would gain an improved understanding of mosquito population growth in relation to local resource availability. Especially encouraging, would be the use of sugar-based control methods that target males to suppress the local population growth.

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